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# Dizertační práce

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Nové metody klasifikace a prognostifikace u paragangliomů a pheochromocytomů

*Fenotypově-genotypové nálezy a řešení paragangliomů hlavy a krku*

New methods of classification and prognosis in paragangliomas and pheochromocytomas

*Phenotypic-genotypic patterns and management of head and neck paragangliomas*

Školitel: doc. MUDr. Martin Chovanec, PhD, MHA

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**Key words: head and neck paragangliomas, surgery,** *SDHD* gene, *SDHB* gene, genetic counselling, germline mutation, somatic mutation

**DEDICATION**

***This PhD thesis is dedicated to my parents. I hereby extend my inestimable gratitude to my parents for being my pillar of support and relentless encouragement throughout the course and always.***

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **CBPGL** | Carotid body paraganglioma |
| **CT** | Computed tomography |
| **CgA** | Chromogranin A |
| **HIF-2α** | Hypoxia Inducible Factor 2, Alpha Subunit |
| **HNPGL** | Head and Neck paraganglioma |
| **IDH2** | Isocitrate Dehydrogenase 2 |
| **JPGL** | Jugular paraganglioma |
| **MET** | Metanephrine |
| **MRI** | Magnetic Resonance Imaging |
| **3-MT** | 3-Methoxytyramine |
| **NF1** | Neurofibromin 1 |
| **NF1 syndrome** | Neurofibromatosis 1 syndrome |
| **NGS** | Next Generation Sequencing |
| **NMET** | Normetanephrine |
| **PCR** | Polymerase Chain Reaction |
| **PET** | Positron emission topography |
| **18F-FDG PET** | 2-deoxy-2-[fluorine-18]fluoro-D-glucose |
| **PPGL** | Pheochromocytoma and paraganglioma |
| **SDH** | Succinate Dehydrogenase |
| **SDHA** | Succinate Dehydrogenase Complex, Subunit A |
| **SDHAF2** | Succinate Dehydrogenase Complex Assembly Factor 2 |
| **SDHB** | Succinate Dehydrogenase Complex, Subunit B |
| **SDHC** | Succinate Dehydrogenase Complex, Subunit C |
| **SDHD** | Succinate Dehydrogenase Complex, Subunit D |
| **SNP** | Single nucleotide peptide |
| **TMEM127** | Transmembrane Protein 127 |
| **TPGL** | Tympanic paraganglioma |
| **VHL** | Von Hippel-Lindau Tumor Suppressor |
| **VPGL** | Vagal paraganglioma |
| **WES** | Whole Exome Sequencing |
| **W+S** | Wait and scan |

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# 1. INTRODUCTION

## 1.1 Introduction to pheochromocytoma-paragangliomas

Paragangliomas and Pheochromocytomas (PPGLs) are rare slow growing hypervascular neuroendocrine tumors of paraganglial cell clusters, embryologically derived from neural crest cells that have comigrated with autonomic nervous system (Corssmit & Romijn, 2014). These tumors are usually benign in nature. They can be associated with either the sympathetic tissue in adrenals as pheochromocytomas (PHEOs) and extra-adrenal locations as sympathetic paragangliomas (sPGLs) (Whalen et al., 1992) or parasympathetic tissue of the head and neck as head and neck paragangliomas (HNPGLs). Extra-adrenal pheochromocytomas may arise in any portion of the paraganglionic system, however they most commonly occur in pre-aortic and paravertebral sympathetic plexus (Whalen et al., 1992). The second most common site is the head and neck. These tumors are classified by the World Health Organization (International Agency for Research on Cancer, 2017; Taïeb et al., 2014).

## 1.2 Head and neck paragangliomas

HNPGLs represent about 3% of all head and neck tumors with an estimated incidence of 1-30/100 000 per year (Wasserman & Savargaonkar, 2001). Only 1-3% of HNPGLs are functional or catecholamine secreting (Offergeld et al., 2012). These tumors may occur at any age, although vast majority of patients become symptomatic between fourth and seventh decade of life. There is a 3-4:1 female predominance (Boedeker, 2011). Such tumors can occur sporadically or as part of genetic syndromes. It has also become apparent that about 30-35% of sporadic tumors are due to a germline mutation, otherwise known as ‘occult familial’ cases (Boedeker et al., 2014; Heesterman et al., 2013; Neumann et al., 2002).

### 1.2.1 Prevalence, localization and characteristics of HNPGLs

Nomenclature of HNPGLs is based on the site of origin (International Agency for Research on Cancer, 2017; Sobol & Dailey, 1990). These paragangliomas arise preferentially from paraganglia of the carotid body giving rise

to carotid body paragangliomas (CBPGLs). Other favorable locations include the dome of jugular bulb (jugular paragangliomas; JPGLs), alongside the tympanic branch of the glossopharyngeal nerve (tympanic paragangliomas; TPGLs) as well as the nodose ganglion of the vagus nerve (vagal paragangliomas; VPGLs) (Boedeker, 2011). Carotid body paragangliomas represent the most common tumor type (up to 60%). This tumor typically presents as a painless, slowly enlarging mass in the lateral part of the neck (Naik et al., 2013; Sajid et al., 2007). Dysphagia, dysphonia, dysarthria due to deficits of the IX-XI, XII cranial nerves, as well as Horner syndrome and syncope may be seen with increasing tumor size. Bilateral tumors are observed in 10% of patients and are usually associated with the familial forms of the disease, especially paraganglioma syndrome type 1 (PGL1). Malignant forms occur in about 6-12% (Erickson et al., 2001; Patetsios et al., 2002; Robertson et al., 2019; Suárez et al., 2006).

Other frequently detected HNPGLs include temporal bone (jugulotympanic; <35-40%) and vagal (<5%) tumors. Patients with such HNPGLs range from asymptomatic to suffering from conductive hearing loss, pulsatile synchronous tinnitus, vestibular problems as well as cranial nerve palsies of mainly IX-XI, XII and VII (Erickson et al., 2001; Smith et al., 2017). Malignancy rates of jugulotympanic PGs is reported at 5.1% (Rinaldo et al., 2004).

In vagal tumors, 75% of cases have a painless lateral neck mass and 55% pharyngeal bulging mass, but 100% have pulsation. Vocal cord palsy leading to hoarseness is present in 28-54% of the cases. Other symptoms such as pulsatory tinnitus, additional cranial nerve deficits (17% in XII, 11% in IX, 6% in X), and Horner syndrome can occur as well (Biller et al., 1989). The risk of malignancy is stated as 6-19% (Hamersley et al., 2016).

Sinonasal, parotid gland, cervical sympathetic chain, laryngeal, esophageal, thyroid and parathyroid, facial nerve, orbital paragangliomas are extremely rare forms of HNPGLs (Barnes, 1991; Langerman et al., 2012; von Dobschuetz et al., 2015; Watson, 1988). Sinonasal PGLs account for the highest malignancy rate (approximately 24%) amongst HNPGLs (Rinaldo et al., 2004).

There are no accepted histopathological or immunohistochemical criteria for the diagnosis of malignant paragangliomas. The only evidence of a malignancy is the

presence of local or distant metastasis that is, presence of paraganglial cells in non-neuroendocrine tissue.

### 1.2.2 Approach to a patient with HNPGL(s)

A multidisciplinary approach is always required to manage such patients. Based on the clinical phenotype, up to 14 medical and surgical specialties maybe involved. However, current practice guidelines recommends mandatory examination with otorhinolaryngology, endocrinology, radiology and clinical genetics. Ideally all departments should be part of the same multidisciplinary team (Lloyd et al., 2020).

#### *1.2.2.1 History, clinical examination and investigations*

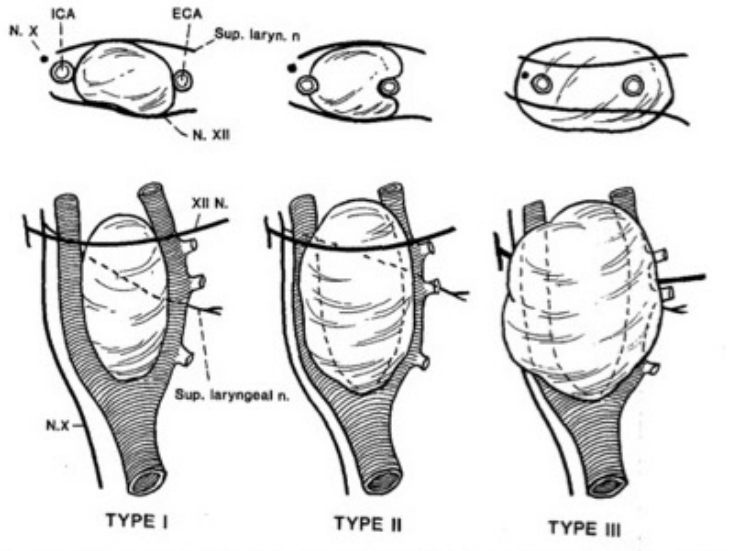
Complete medical and family history should be completed. Family history plays a significant role in disease predilection since most hereditary forms are of autosomal dominant in nature. Although, a vast majority with HNPGLs are biochemically silent, in patients with hypertension, this carries a high degree of importance. Most patients can be asymptomatic; therefore, it should also be noted that incidental finding of these tumors is not an uncommon phenomenon.

Current standardized practice recommends a comprehensive radiological investigation that should include anatomical and functional imaging. The former uses computed tomography (CT) or the preferred magnetic resonance imaging (MRI) (Lenders et al., 2014) . CT scanning assesses bone integrity, hence evaluates the degree of bone destruction in the base of the skull. Whereas MRI is used in detailed analysis of soft tissues, dural invasion and intradural spread. This method of examination also shows a classic “salt and pepper” sign due to the high vascularity of HNPGLs. It should also be mentioned that with the advancement of radiological techniques, CT/MR Angiography has replaced the conventional Digital Subtraction Angiography. This not only helps in identifying uncertain site of tumor origin due to the hypervascular character but also assists in interventional radiology such as pre-operative embolization. Functional imaging is performed with PET/CT (Crona et al., 2017; Santhanam & Taïeb, 2014). This is a key method

that determines neuroendocrine origin, presence of multiple tumors as well as metastasis.

Different classification systems were suggested to evaluate both extent of disease as well as to help with planning of tumor management. The Shamblin classification is used as an important predictor of vascular morbidity during surgical resection (Figure 1) and is based on MRI. This describes the relationship of the tumor to the external, internal and common carotid arteries (Shamblin et al., 1971). Type I tumors are localized and easily resected. Type II includes tumors adherent or partially surrounding internal and external carotid arteries. Type III paragangliomas intimately surround or encase the vessels. In 2006, Luna-Ortiz introduced a small subcategory to Type III (Luna-Ortiz et al., 2006). Here Type IIIa represents the old Type III, whilst IIIb includes tumors of any type (I,II or III) with infiltration of the vessel wall and not just circumferential encasement.

Figure 1. Shamblin's classification of the difficulty in surgical resection of CBPGLs *(partially adapted from Shamblin et al., 1971)*



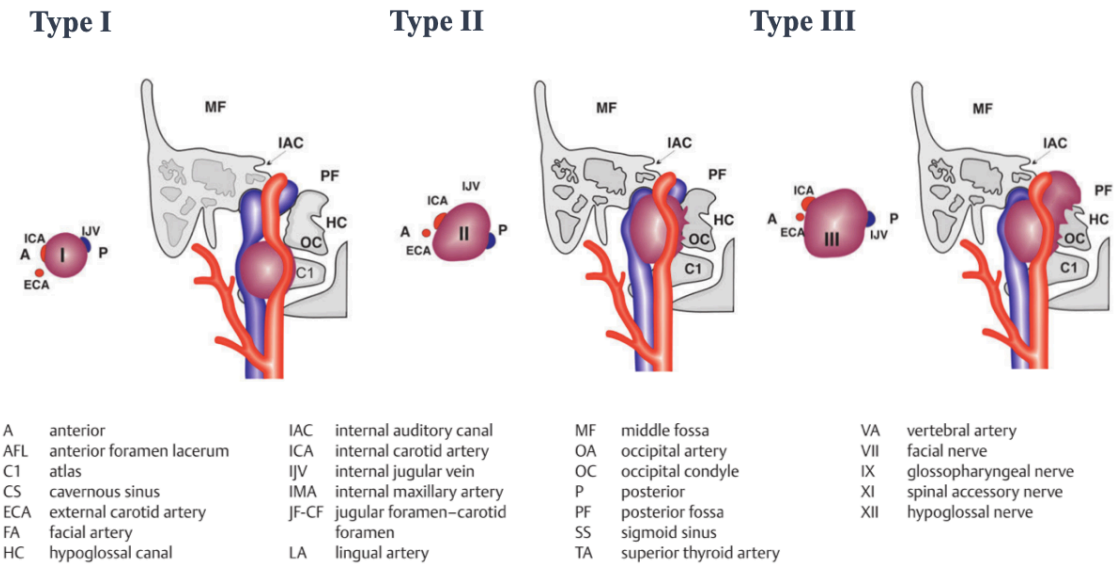

Tympanojugular tumors have been categorized by many authors. With the earliest descriptions since 1949, lateral skull base glomus tumors were classified as tympanicum and jugulare lesions (Alford & Guilford, 1962; Bickerstaff & Howell, 1953; Guild, 1953; Lundgren, 1949). With improvements in imaging and surgical technique, it became apparent that a more detailed classification scheme was required. In 1978, Fisch devised the first practical classification system (types A to

D) whereby tumors could be classified by area of involvement and treated with particular consideration directed toward intrapetrous ICA involvement (type C) and intracranial extension (type D) (Fisch, 1978). In 1982, Jackson et al introduced a new classification for paragangliomas of the temporal bone (I-IV) to facilitate management of extensive lesions (Jackson et al., 1982), stressing the importance of collaboration between the neurotologist and neurosurgeon. Subsequently, Fisch's classifications were used to classify both jugulotympanic and vagal tumors (Table 1; Figure 2) as it indicates the extent of temporal bone and skull base destruction which in turns dictates the extent of surgical approach needed for safe management (Fisch, 1978; Fisch & Mattox, 1988; Offergeld et al., 2012).

Table 1. Classification of jugular paragangliomas according to Fisch *(partially adapted from Fisch, 1978)*

| | |
|---|---|
| A | Limited to glomus tympanon |
| B | Limited to tympanomastoid area with/without erosion of jugular bulb |
| C | Involvement and destruction of infralabyrinthine and apical compartments |
| $D_1$ | Intracranial extension < 2 cm in greatest diameter |
| $D_2$ | Intracranial extension > 2 cm in greatest diameter |
| $D_3$ | Inoperable intracranial extension |

Figure 2. Fisch's classification of vagal PGLs *(partially adapted from Sanna et al., 2013)*



Later, Green et al proposed a novel classification system (1–7) with associated surgical approaches that officially recognized the issue of intradural extension of the disease (Green et al., 1994). The classification of tympanojugular paragangliomas given by Fisch et al. in 1988 was modified by Shin et al in 2012 (Shin et al., 2012). This modified Fisch classification is used currently in most clinical practices (Table 2).

Table 2. Modified Fisch classification of temporal bone paragangliomas *(partially adapted from Shin et al., 2012)*

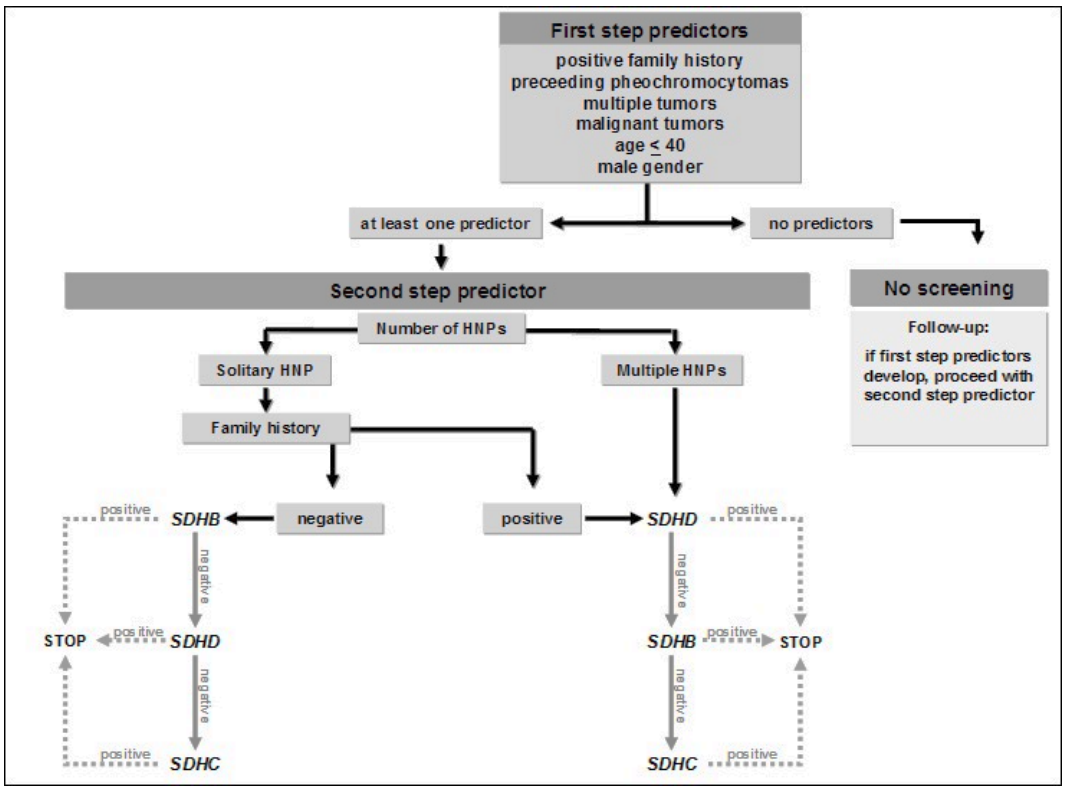
| | | |
|---|---|---|
| Tympanomastoid paragangliomas | Class A | Tumors limited to the middle ear |
| | A1 | Tumors completely visible on otoscopic examination |
| | A2 | Tumor margins are not visible on otoscopy. Tumors may extend anteriorly to the eustachian tube and/or to the posterior mesotympanum |
| | Class B | Tumors limited to the tympanomastoid area without destruction of bone in the infralabyrinthine compartment of the temporal bone |
| | B1 | Tumors confined to the middle ear cleft with extension to the hypotympanum |
| | B2 | Tumors involving the middle ear cleft with extension to the hypotympanum and the mastoid |
| | B3 | Tumors confined to the tympanomastoid compartment with erosion of the carotid canal |
| Tympanojugular paragangliomas | Class C | Tumors extending, destroying bone of the infralabyrinthine and apical compartment of the temporal bone and involving the carotid canal |
| | C1 | Tumors limited with limited involvement of the vertical portion of the carotid canal |
| | C2 | Tumors invading the vertical portion of the carotid canal |
| | C3 | Tumors with invasion of the horizontal portion of the carotid canal |
| | C4 | Tumors reaching the anterior foramen lacerum |
| | Class D | Tumors with intracranial extension |
| | De1 | Tumors up to 2 cm dural displacement |
| | De2 | Tumors with more than 2 cm dural displacement |
| | Di1 | Tumors up to 2 cm intradural extension |
| | Di2 | Tumors with more than 2 cm intradural extension |
| | Di3 | Tumors with inoperable intradural extension |
| | Class V | Tumors involving the VA |
| | Ve | Tumors involving the extradural VA |
| | Vi | Tumors involving the intradural VA |

Biochemical tests are another important aspect of examination. These should be performed to attain the functional activity of tumors. Plasma metanephrines and methoxytyramine (a metabolite of dopamine) in particular indicate biochemically active tumors and should be tested. It can also be combined with urine metanephrines (van Duinen et al., 2013). Furthermore, Chromogranin A, a biomarker, is also released by neuroendocrine tissues along with catecholamines. It is a more sensitive and specific diagnostic tool in detecting pheochromocytomas (familial and sporadic) rather than paragangliomas (Hsiao et al., 1990).

Genetic analysis and counselling for the index patient should be given priority. Predictors for the hereditary form of the disease should be considered as young age (<40 years), positive family history of PPGLs as well as multiple tumors

including bilateral carotid body tumors. It is advised to exclude $SDH_x$ mutations beginning with *SDHD* followed by *SDHB* and then *SDHC* gene mutations (Crona et al., 2017; Neumann et al., 2009; Smith et al., 2017). If malignancy is suspected, *SDHB* should be tested first (Neumann et al., 2004) (Figure 3). If an index patient is positive then carriers should be identified amongst first degree relatives (Muth et al., 2018).

Figure 3. An example of cost-effective genetic analysis *(adapted from* Smith et al., 2017)




*1.2.2.2 Treatment options, risks and complications*

Several approaches have been advocated in the management of HNPGLs. The choice of treatment should be dependent on patient-related and tumor factors. The 2 main aims of any treatment approach are long-term tumor control and minimal cranial nerve morbidity.

HNPGLs are typically slow growing tumors (1-2mm/year) (Jansen et al., 2000), hence the 'wait and scan' approach can be used for asymptomatic cases with low risk of malignancy and ideally in the absence of germline mutation. These patients should be monitored very well with annual MRI scans, otherwise there is always a risk of irreversible complications from tumor infiltration.

Surgical resection with or without preoperative embolization can be curative for most patients with HNPGLs, however size and localization should be respected mainly due to the risk of postoperative cranial nerve morbidity and eventual vascular complications. Most importantly, with improvements in preoperative management of the patients, the expected risk of stroke has reduced from 30% to less than 3% and mortality rates are nearly negligible during carotid paraganglioma surgery (Hudgins, 2017).

The second most common treatment strategy of active treatment is radiotherapy as paragangliomas are deemed to be radiosensitive. Conventionally fractioned external-beam radiotherapy has been used for several decades however one of rising interest is image-guided radiosurgery or stereotactic surgery. External-beam radiation technique carries a small risk of adverse effects (Krych et al., 2006); however, radiosurgery uses a more precise form of radiation and with recent advances, has low rate of side effects (Ibrahim et al., 2017). And although radiosurgery is being recommended as frontline therapy due to the negligible risk of postoperative cranial nerve morbidity, one grave concern of using radiotherapy is the long-term effects of developing malignant tumors (Krych et al., 2006), hence should be used with caution amongst young patients. Radiotherapy also has no role in secreting tumors.

The third option is the use of targeted therapy (Taïeb et al., 2014). Although it is promising, efficacy and long-term tumor control with HNPGLs are yet to be established.

It should be noted that managing multiple HNPLs is considerably challenging, if surgery is considered, it is recommended to remove the larger tumor first. The aim is always a stepwise approach, thus avoiding bilateral cranial nerve deficits and irreversible disabilities. Therefore, an alternative is advocating a combination regime. Furthermore, due to the lack of specific histopatholological criteria, it is difficult to accurately diagnose and treat malignant forms of these tumors. However, with the advent of advanced molecular genetics, predilection and behavior of tumors can be determined.

## 1.3 Genetic background in PPGLs

These tumors can occur sporadically or as a part of hereditary syndromes (Boedeker et al., 2014; Else et al., 1993; Martucci & Pacak, 2014; Piccini et al., 2012). Hereditary tumors can be suspected in case of familial antecedents of the disease (Latif et al., 1993), multiple tumors and early onset of the disease. Whilst patients older than 40–50 years (Neumann et al., 2009) are usually diagnosed with sporadic tumors. Until recently, it was considered that only 10% of PPGLs, predominantly pheochromocytomas, were associated with certain hereditary syndromes namely von Hippel–Lindau disease, multiple endocrine neoplasia type 2, and neurofibromatosis type 1, resulting from a germline mutation in tumor suppressor gene *Von Hippel Lindau (VHL)*, *proto-oncogene RET* (Eng, 1996), and tumor suppressor gene *Neurofibromatosis1 (NF1)* (White & O'Connell, 1991) respectively. It has now become evident that nearly 35% of suspected sporadic PPGLs are due to a germline mutation in one of the susceptible genes (Boedeker et al., 2014; Neumann et al., 2002). These are as follows the four subunits of the *Succinate Dehydrogenase (SDHx*) complex (e.g. *SDHA, SDHB, SDHC, SDHD*) (Astuti et al., 2001; Baysal et al., 2000; Burnichon et al., 2010; Niemann & Müller, 2000), *succinate dehydrogenase complex assembly factor 2 (SDHAF2)* (Bayley et al., 2010), *VHL* (Latif et al., 1993), *proto-oncogene RET* (Eng, 1996), *NF1* (White & O'Connell, 1991), *transmembrane protein 127 (TMEM127*) (Qin et al., 2010), *MYC-associated factor X (MAX)* (Comino-Méndez et al., 2011), *Fumarate Hydratase (FH)* and *Malate Dehydrogenase 2 (MDH2)* (Castro-Vega et al., 2015). In addition, germline as well as somatic mutations of other genes (Gimenez-Roqueplo et al., 2012) such as *kinesin family member 1B (KIF1b)* (Schlisio et al., 2008), *EGLN1/prolyl hydroxylase 2 (PHD2)* (Ladroue et al., 2008), *isocitrate dehydrogenase 1* (*IDH1)* (Gaal et al., 2010), *hypoxia-induced factor 2 alpha (HIF-2α)* (Zhuang et al., 2012), *HRAS* (Oudijk et al., 2014) were also reported.

### 1.3.1 Hereditary factors and tumorigenesis

Many large-scale genomic analyses have been conducted worldwide on independent series over the last few years. These include single nucleotide polymorphism (SNP) array and comparative genomic hybridization, mRNA and

microRNA (miRNA) expression studies (de Cubas et al., 2013) and DNA methylation profiling (de Cubas et al., 2015; Letouzé et al., 2013). These approaches have led to the description of well-defined tumorigenic pathways and their corresponding tumor subtypes (Burnichon et al., 2016).

Gene expression profiling showed that PPGLs could be separated by unsupervised analysis into two main clusters: cluster 1 (C1) and cluster 2 (C2) based on transcriptomes (Corssmit & Romijn, 2014; Crona et al., 2013; Dahia et al., 2005; Eisenhofer et al., 2004; López-Jiménez et al., 2010). DNA methylation and miRNA profiling showed a major influence of the main genetic drivers on the somatic molecular phenotype (Castro-Vega et al., 2015; de Cubas et al., 2013, 2015; Letouzé et al., 2013).

*1.3.1.1 Cluster 1-related tumors*

Cluster 1 is further subdivided into 2 groups. In C1A, DNA and histone hypermethylation is seen in *SDHx*, *FH* and *MDH2* gene linked tumors (Bayley et al., 2010; Burnichon et al., 2011); whilst in C1B, glycolysis is activated in *VHL* related tumors and *hypoxia-inducible factor 2-alpha (HIF-2α)* (Burnichon et al., 2011, 2016; López-Jiménez et al., 2010; Zhuang et al., 2012). Germline or somatic mutations are characterized by transcription signatures indicating reduced oxidoreductase activity and thus increased angiogenesis and hypoxia (Dahia et al., 2005; Favier et al., 2009).

According to Knudson's two-hit theory, a heterozygous germline mutation in *SDHx* tumor suppressor genes are usually associated with loss of heterozygosity in the tumor. This results in inactivation of SDH enzymatic activity and leads to accumulation of succinate, and thereby inhibits prolyl hydroxylase domain (PHD) enzymatic activity. PHDs represent enzymes crucial for the degradation of HIF. Thus, even in the presence of oxygen, HIF cannot be degraded via proteasome-mediated degradation driven by VHL protein. This induces angiogenesis and tumorigenesis (Favier et al., 2009; Gimenez-Roqueplo et al., 2012). The latter also happens in *VHL* mutations. Interestingly, a hypermethylator phenotype in *SDH-related* paragangliomas has also been described. Such tumors accumulate succinate, which inhibit 2-oxoglutarate-dependent histone and DNA demethylase

enzymes, resulting in epigenetic silencing, thereby affecting neuroendocrine differentiation (Corssmit & Romijn, 2014; Letouzé et al., 2013).

*Succinate dehydrogenase (SDH) mutations - according to chronological order*

*SDHD*

In the year 2000, Baysal et al. discovered *SDHD* mutations in families with paraganglioma syndrome type 1 (PGL1) and also revealed that germline mutations in the *SDHD* gene are located at 11q23.18 (Baysal et al., 2000). This led to understanding the molecular mechanism of inheritance of paragangliomas. Later in 2009, Pasini and Stratakis extensively reviewed 95 international manuscripts on *SDHx* mutations, that comprised a total of 395 *SDHD* mutation carriers (Pasini & Stratakis, 2009). Other case series studies have also described the genotype-phenotype correlation of *SDHD* mutations (Burnichon et al., 2009; Hensen et al., 2010, 2011; Neumann et al., 2009; Papaspyrou et al., 2012; Schiavi et al., 2005). The key feature of this syndrome is presence of multiple (synchronous or metachronous) tumors which are seen in nearly 60%-79% of affected patients. HNPGLs affect about 91-98% of patients with PGL1 (Benn et al., 2006; Boedeker et al., 2014; Burnichon et al., 2009; Gimenez-Roqueplo et al., 2012; Neumann et al., 2009; Pasini & Stratakis, 2009), whilst the risk of developing sympathetic paragangliomas ranges from 16 to 60% (Boedeker et al., 2014; Burnichon et al., 2009; Neumann et al., 2009). *SDHD*-related HNPGLs are vastly biochemically silent, that is, with the exception of 20%, that secrete dopamine and/or its metabolite methoxytyramine, which can be useful for monitoring these patients (van Duinen et al., 2013). The inheritance pattern in PGL1 families exhibit a peculiar inheritance pattern with a distinct "parent-of-origin dependent effect" (van Schothorst et al., 1998). Although, *SDHD* mutations can be inherited both via the maternal and paternal lines, however paragangliomas almost never develop after maternal transmission (Hensen & Bayley, 2011; Pigny et al., 2008; van der Mey et al., 1989). PGL1 can seem to skip generations, as maternally derived *SDHD* mutation carriers will still pass the mutation to their offspring in 50% of cases. This partly explains the occurrence of *SDHD* germline mutations in apparently nonfamilial cases.

Imprinting of *SDHD* has never been established, and the fact that this inheritance pattern is also found in PGL2 families (linked to the SDHAF2 gene, located on 11q13), but not in other PGL syndrome families, suggests that other factors related to chromosome 11 contribute to this phenomenon (Boedeker et al., 2014).

Age-related penetrance in *SDHD* mutation showed 50% penetrance by age 31 rising to 87% by the age of 70 (Hensen et al., 2010; Neumann et al., 2004). The average range of age at diagnosis of *SDHD*-linked tumors is 25 to 38 years (Hensen et al., 2010). HNPGLs affect about 40% of patients with PGL1 with median age at the time of diagnosis being 40 years (Boedeker et al., 2014; Letouzé et al., 2013; Martucci & Pacak, 2014; Neumann et al., 2009). Pheochromocytomas can affect about half of the PGL1 patients and as such present at earlier age than HNPGLs (Boedeker et al., 2014). The risk of malignant paragangliomas in *SDHD* mutation carriers showed that the prevalence of malignant tumors varied widely, ranging from 0% to up to 23%, with pooled incidence for malignant tumors of 8% (van Hulsteijn et al., 2012).

*SDHC*

Familial paraganglioma type 3 (PGL3) is related to mutations of *SDHC*, that is located on chromosome 1q21 (Baysal et al., 2004; Niemann & Müller, 2000). In comparison to PGL1 and PGL4, PGL3 is rare and mostly present with single HNPGLs (Burnichon et al., 2009; Neumann et al., 2009; Schiavi et al., 2012). Multiple HNPGLs are found only in about 19% to 31% of patients. Two large series of patients showed similar prevalence of 3.6% and 4.3% for *SDHC* mutations respectively (Burnichon et al., 2009; Neumann et al., 2009). However, risk of developing HNPGLs with PGL3 is as high as 100% (Baysal et al., 2004; Boedeker et al., 2014; Schiavi et al., 2005). Family history is positive in 12-25% of patients with *SDHC*, indicative of low tumor penetrance (Schiavi et al., 2005). The average age at diagnosis is higher than for the other paragangliomas (about 38 to 46 years) (Schiavi et al., 2005). Pheochromocytomas, extra-adrenal and malignant paragangliomas are rarely reported (Baysal et al., 2004; Burnichon et al., 2009; Neumann et al., 2009; Niemann et al., 2003; Schiavi et al., 2005).

*SDHB*

Patients with familial paragangliomas showing mutations of *SDHB* was located on chromosome 1p36.13. This mutation is related to paraganglioma syndrome type 4 (PGL4) (Astuti et al., 2001; Boedeker et al., 2014). Those with *SDHB* mutations frequently develop sPGLs (52% to 84%) followed by PHEOs (18% to 28%). In comparison to PHEOs and HNPGLs, extra-adrenal sPGLs have long been known to have a greater predisposition to malignancy (Proye et al., 1992). It is not clear whether this effect is the result of location, variant status, or a combination of both (Ricketts et al., 2010). The risk of developing HNPGLs with PGL4 is 27-31% (Neumann et al., 2009). Multifocal HNPGLs are also significantly less frequent (around 8%) (Boedeker et al., 2014; Klein et al., 2008; Neumann et al., 2009; Pasini & Stratakis, 2009). The estimated age-related tumor penetrance in carriers is 29% at age 30 rising to 45% at age 40 years (Benn et al., 2006). PHEOs and sPGLs usually present earlier than HNPGLs. A high percentage of cases (20.6% up to 41%) are associated with malignant PHEOs and HNPGLs (Boedeker et al., 2014; Klein et al., 2008; Neumann et al., 2009; Pasini & Stratakis, 2009). Therefore, it is a well-established negative prognostic factor (Amar et al., 2007).

It has also been shown that those with a germline *SDHB* pathogenic variant can develop malignant disease at any paraganglion site (Benn et al., 2006; Gimenez-Roqueplo et al., 2003; López-Jiménez et al., 2010; Neumann et al., 2004). The risk of malignant PPGLs in *SDHB* revealed a pooled incidence of 17%. The pooled risk in prevalence studies, ranged from 13% to 23% in the *SDHB* group. Although, it was concluded that incidence and prevalence of malignant HNPGLs and PHEOs are higher in *SDHB* carriers (van Hulsteijn et al., 2012), risk of malignancy in sPGLs is even higher. Moreover, a multivariate analysis of 54 patients with malignant PPGLs done in France demonstrated that identification of *SDHB* mutation was not only seen in younger patients and more frequently associated with extra-adrenal tumors but was also independently correlated with mortality. The 5-year survival probability significantly reduces to 36% for patients with *SDHB* mutation versus 67% for patients without *SDHB* mutation (Amar et al., 2007).

*SDHAF2*

The *SDHAF2* gene responsible for paraganglioma syndrome type 2 (PGL2) was identified in 2009 (Hao et al., 2009), located on chromosome 11q13.1 (Boedeker et al., 2014). SDHAF2 affects flavination of SDHA. A follow-up multicenter study was undertaken in Spain and the Netherlands with the joint aims of identifying new mutation carriers and assessing the frequency of *SDHAF2* mutations in 443 sporadic PPGLs showed that all currently affected mutation carriers were identified to be affected exclusively with HNPGLs mutations. Therefore, this gene makes a very modest contribution to the overall genetic burden in these syndromes (Bayley et al., 2010). Only one additional *SDHAF2*-related family was identified in Spanish patients, which interestingly carried the exact mutation, p.Gly78Arg, previously discovered in the Netherlands. Nevertheless, a familial relationship to the Dutch kindred was excluded (Baars et al., 1981; Bayley et al., 2010; Gimenez-Roqueplo et al., 2012; Hensen & Bayley, 2011). The same study also concluded that *SDHAF2* mutation analysis is justified in very young patients with isolated HNPGLs in the absence of *SDHD, SDHC*, or *SDHB* mutations and in individuals with familial antecedents who are negative for mutations in all other risk genes. Another exclusive study done in Netherlands over a 30-year period followed the Dutch kindred, originally described in 1981 (Baars et al., 1981), with PGL2 showing frequent occurrence of HNPGLs (Kunst et al., 2011). Amongst 3 generations, 72 living family members were traced and linkage analysis was carried out in 57 members. The findings associated with the same mutation were below 40 years at diagnosis, 100% affected at age 50, 100% with HNPGLs and 91% with multifocality (Kunst et al., 2011). Accordingly, the risk of developing HNPGLs with SDHAF2 can also be up to 100% (Baars et al., 1981; Bayley et al., 2010; Kunst et al., 2011; Neumann et al., 2009; Rinaldo et al., 2004).

*SDHA*

The final subunit in the mitochondrial complex, SDHA is located on chromosome 5p15. Mutations of SDHA lead to paraganglioma syndrome type 5 (PGL5) that was discovered in 2010 (Burnichon et al., 2010). HNPGLs have been

detected with mutations in *SDHA* (Vicha et al., 2013), however the accurate risk of developing such tumors remains unfamiliar.

*von Hippel-Lindau (VHL)*

The inactivation of the tumor suppressor gene *VHL* located on chromosome 3p25-p26 leads to von Hippel-Lindau syndrome (Latif et al., 1993). The VHL protein regulates the activity of hypoxia-inducible factor-alpha (HIF-α) and cellular processes, including angiogenesis. The syndrome is characterized by a predisposition to multiple tumor types and can be classified on the risk of developing PPGLs. VHL type 1 are predisposed to retinal angiomas, central nervous system hemangioblastomas and renal carcinomas. VHL type 2, specifically subtype 2C, predisposes to only PPGLs without any type 1 tumors. The age of onset of PPGLs is approximately 30 years, though patients as young as 5 years have been reported (Gimenez-Roqueplo et al., 2012; Karasek et al., 2013). One study detected somatic mutations of the *VHL* gene in about 14% of sporadic PPGLs (Burnichon et al., 2011). The prevalence of hereditary HNPGLs was 5 out of 1,000 in patients with *VHL* mutation (Boedeker et al., 2009; Offergeld et al., 2012).

*Hypoxia-inducible factor 2-alpha (HIF-2α)*

Locus of this gene was identified as 2p21–p16 (Dahia et al., 2005). Novel somatic mutations in the gene encoding HIF-2α in 2 patients were identified in Bethesda in 2012. One patient presented with paraganglioma and the other with paraganglioma and somatostatinoma, both had polycythemia, suggesting the existence of a new syndrome (potentially to be named Pacak–Zhuang syndrome) (Zhuang et al., 2012). This was also confirmed in 4 female patients of different ethnicities (Pacak et al., 2013). A novel *HIF-2α* germline mutation was also reported later (Lorenzo et al., 2013). Hypoxia-inducible factors are transcription factors controlling energy, iron metabolism, erythropoiesis, development, glycolysis, and other cell functions. The longer half-life of the mutant HIF-2α protein resulted in the upregulation of downstream HIF-2α targets (endothelin-1, erythropoietin, glucose transporter 1, and vascular endothelial growth factor). This is currently believed to be the pathogenic mechanism that leads to tumor

development (Zhuang et al., 2012). Only one patient was diagnosed with jugular paraganglioma on a follow up $^{18}$F-DOPA PET/CT scan (Pacak et al., 2013), otherwise no other HNPGLs have been described.

*Fumarate hydratase (FH) and malate dehydrogenase 2 (MDH2)*

FH and MDH2 are two enzymes that belong to the tricarboxylic acid cycle (Burnichon et al., 2016). The incidence of *FH* mutation in PPGLs is estimated at about 1%. Mutations in *FH* predispose to hereditary cutaneous and uterine leiomyomatosis and type 2 papillary renal cell carcinoma. Approximately 40% of cases carrying germline *FH* mutation presented with metastatic disease (Burnichon et al., 2016) and only one germline *MDH2* mutation has been identified in a patient with multiple malignant PPGLs (Burnichon et al., 2016). No cases of HNPGLs have been reported.

*Prolyl hydroxylase domain-containing protein 2 (PHD2)*

The product of the *PHD2* gene is a prolyl hydroxylase that is known to function in hydroxylation of hypoxia-inducible factor alpha and thus function in the cells´ response to hypoxia (Ivan et al., 2002). So far only one study described one patient with congenital erythrocytosis and recurrent abdominal PGLs, that was found to harbor a mutation in the *PHD2* gene (Ladroue et al., 2008). Nevertheless, no HNPGLs were found.

*1.3.1.2 Cluster 2-related tumors*

The gene expression signatures of these tumors includes genes that mediate translation initiation, protein synthesis, adrenergic metabolism, neural/neuroendocrine differentiation and abnormal activation of kinase signaling pathways such as RAS/RAF/MAPK and PI3K/AKT/mTOR. The genes in in this cluster are *RET* proto-oncogene (Eng, 1996), *NF1* (White & O'Connell, 1991), *TMEM127* (Qin et al., 2010), *MAX* (Comino-Méndez et al., 2011) and *H-RAS* (Burnichon et al., 2016; Crona et al., 2013).

*Receptor tyrosine-protein kinase (RET) proto-oncogene*

The RET protein is a receptor tyrosine kinase located on chromosome 10q11.2 (Boedeker et al., 2014). It regulates cellular proliferation and apoptosis. *RET* mutations have been associated with increased activation of PI3K/v-Akt signals and RAS/RAF/MAPK signaling pathways (Eng, 1996; Vicha et al., 2013), which leads to the development of Multiple endocrine neoplasia type 2 (MEN2). Medullary thyroid cancer (MTC) is the most common condition and usually first to be diagnosed in MEN2 patients. MEN2 have a 30-50% chance of developing pheochromocytoma. Patients with familial MTC, a subtype of MEN2, do not have a risk of developing PHEOs (Boedeker et al., 2014). Both somatic and germline mutations have been reported (Martucci & Pacak, 2014). One study detected somatic mutations of the *RET* gene in about 14% of sporadic PPGLs (Burnichon et al., 2011). It should be noted that HNPGLs do not develop without other MEN2-associated manifestations (Boedeker et al., 2009). HNPGLs have only been described in 3 patients with MEN2. Carriers can be detected in early life due to strong family history.

*Neurofibromatosis type 1 (NF 1)*

The *NF1* gene, located on chromosome 17q11.2, encodes a GTPase activating protein. It is involved in multiple signaling cascades, important to cellular growth and differentiation (White & O'Connell, 1991). The gene mutation leads to the neurofibromatosis type 1 syndrome (Wallace et al., 1990). Hallmark of the syndrome is dominated by multiple neurofibromas of the skin, cafe-au-lait spots, axillary freckling, and Lisch nodules of the iris (Boedeker et al., 2014). In addition, other tumors such as MTC and carcinoids can also be seen (Martucci & Pacak, 2014). In comparison to VHL and MEN2 syndromes, the rate of PHEO/PGL development in NF1 is significantly lower. Initially in 1987, HNPGL was detected in 1 patient with NF1. This syndrome was reported as a new neuroendocrine neoplasia (DeAngelis et al., 1987). Recently, somatic *NF1* mutations have been linked to the pathogenesis of apparently sporadic PPGLs (Burnichon, Buffet, et al., 2012). However, further large international analyses revealed no association with HNPGLs (Bausch et al., 2006; Neumann et al., 2009).

*Transmembrane protein 127 (TMEM127)*

*TMEM127* is a tumor suppressor gene located on chromosome 2q11.2 (Qin et al., 2010). Mutations of *TMEM127* have been related to PHEOs with similar transcriptional profile to *NF1* and *RET* related tumors. However, neither RAS activation nor AKT phosphorylation was seen, indicating that TMEM127 loss is not identical to either NF1 or RET. It has been shown that the mammalian target of rapamycin complex 1 (mTORC1) is specifically affected by TMEM127 knockdown, leading to increased phosphorylation of targets of mTORC1 (Qin et al., 2010; Vicha et al., 2013). Genetic studies of PPGL patients indicate a low prevalence of 2% of TMEM127 mutations (Abermil et al., 2012; Qin et al., 2010). In most cases, *TMEM127* mutation carriers suffered from either unilateral or bilateral PHEOs only and secreted a high level of metanephrines (Abermil et al., 2012). Only one patient with bilateral carotid paragangliomas was reported (Abermil et al., 2012; Offergeld et al., 2012; Qin et al., 2010). A unique finding in these patients was the older average age at presentation, which is similar to sporadic cases.

*MYC-associated factor X (MAX)*

In 2011, *MAX* was identified as a new pheochromocytoma tumor suppressor gene in three independent patients with familial antecedents of the disease (Comino-Méndez et al., 2011). The protein encoded by the *MAX* gene is a member of the basic helix–loop–helix leucine zipper (bHLHZ) family of transcription factors and located on chromosome 14q23.3. *MAX* mutations are associated with bilateral PHEOs and show apparent paternal transmission of the disease (Comino-Méndez et al., 2011). In a large international study, it was confirmed that germline mutations are responsible for PPGLs in 1.12% of cases and somatic mutations were found in 1.65% of cases (Burnichon, Cascón, et al., 2012). No HNPGLs were reported.

*Harvey Retrovirus-associated DNA sequences (H-Ras)*

Constitutive RAS signaling is known to increase cell proliferation and induce tumor formation in many types of cancers (Adari et al., 1988). This is located

on chromosome 11p15.5 (King & Pacak, 2014). Genetic mutations of *NF1* and *RET* are known to affect RAS signaling and are associated with the formation of PPGLs. Though later in 2013, development of tumors was found with mutation in RAS itself (Crona et al., 2013). The presence of *HRAS* somatic mutations in a series of 4 male patients (3 presenting with PHEOs and 1 with an sPGL) was described (Crona et al., 2013). The discovery of this mutation presents a further link between RAS proteins and the development of PPGLs (King & Pacak, 2014), however no HNPGLs were described.

### 1.3.2 Trends of genetic studies of PPGLs in Czech Republic

A large cohort of patients with PPGLs in Czech Republic was reported in 2016. It analyzed 15 years of retrospective data, during which these patients underwent genetic analysis for germline mutation of *SDHD*, *SDHB*, *RET* and *VHL* genes. Furthermore, the presence of BRAF somatic mutation was studied; this is an excellent treatment target for metastatic disease. The patients were consented for examination of somatic mutation. DNA was extracted from peripheral blood samples and from frozen tumor's sample after histological confirmation of PPGL. Following which, the patients were screened for BRAF V600E mutation using direct Sanger sequencing and QRT-PCR (Vosecka et al., 2017). Results showed a cohort of 64 (32 males; 32 females) patients of 7-77 years of age. Only one patient had positive family history. Fifty-four patients were found with pheochromocytomas (single or bilateral) and ten with paragangliomas. Only 3 patients were diagnosed with HNPGLs; two (male of aged 27 and female of 31 years) had primary tumors without family history of PPGLs and one was a 23-year old male with positive family history and neck metastasis. The 27-year old had *SDHD* germline mutation, the most common mutation in patients with HNPGLs (Vosecka et al., 2017). The BRAF V600E mutation was not found in any of the samples. Despite the timeline, only 4.7% of the cohort had HNPGLs.

It should be mentioned that an earlier study reported the surgical treatment of 16 tympanojugular paragangliomas (Skrivan et al., 2010), but genetic analysis was not done. In Czech Republic, there is a lack of studies reporting the frequency and/or prevalence of gene mutations related to HNPGLs. This has in turn, led to a

discrepancy in the contribution of genetic patterns in Europe. More importantly, from a clinical point of view, such findings have an impact on the management and prognosis of such patients in standard practice. Therefore, we, decided to design a prospective study to understand genotypic-phenotypic patterns of patients with HNPGLs in Czech Republic.

## 2. STUDY AIMS AND HYPOTHESES

1. To perform systematic review in order to identify the specific genes involved in paragangliomas, that, when mutated, can directly lead to HNPGLs.
2. To report the genotypic-phenotypic pattern of HNPGLs in our patients and help formulate an algorithm for screening of suspected HNPGLs in Czech Republic.
3. Our study hypotheses will assume that amongst patients who underwent genetic examination:

i. at least 50% of all patients with HNPGLs will be positive for *SDHD* germline mutation.

ii. patients under 40 years of age and/or with bilateral carotid body tumors will have higher affinity for *SDHD* germline mutation.

iii. approximately 30% of apparently sporadic tumors are due to a germline mutation ("occult familial" cases) related to the *SDHD* gene.

## 3. MATERIALS AND METHODS

In order to carry out a thorough systematic review, we performed an extensive literature review of the PubMed database from the National Library using a combination of specific keywords. These keywords were paragangliomas; head and neck paragangliomas; sporadic; hereditary syndromes; germline mutation; somatic mutation; succinate dehydrogenase; SDHD; SDHB; VHL; NF1; TMEM127; HIF; RET. This was done to ascertain research work that provides evidence-based data on gene mutations related to head and neck paragangliomas. The worldwide distribution of incidence of the most frequent mutation was identified. Research papers with data extending up to the 1950s were considered to improve precision of comparative analysis of incidence distribution.

## 3.1 Methodology for genetic testing and validation of hypotheses in HNPGLs

All patients with HNPGLs referred to the department of Otorhinolaryngology, 3rd Faculty of Medicine, Charles University and University Hospital Královské Vinohrady between October 2016 and August 2021 were analyzed. A multidisciplinary approach was adopted for all the patients. After detailed medical and family history, patients underwent standard examination and clinical investigations including biochemical tests, ultrasound of the neck, MRI and/or CT including MR- or CT-angiography according to the localization. PET/CT scan was done in all patients with multiple tumors or suspected metastases. Shamblin's and modified Fisch's classifications were employed to classify the extent of carotid, vagal and jugular PGLs. According to our protocol, all patients who consented underwent genetic testing.

Our first aim of genetic examination was to detect the presence of *SDHD* mutation, the most commonly seen mutation in HNPGLs and the first recommended gene to be tested in benign tumors. In rare cases with suspected malignant tumors, *SDHB* mutation is tested first. Germline mutation status is analyzed using peripheral blood samples, whilst analysis of somatic mutations also requires tumor samples. In our center in Czech Republic, as per protocol, we use Polymerase Chain Reaction (PCR) Sanger sequencing to first exclude *SDHD* germline mutation. This is followed by Next Generation Sequencing (NGS). The latter is used to assess 123 genes (standard panel genes for pheochromocytoma/paraganglioma). However, in certain cases with multiple HNPGLs or if requested by the primary referring physician, NGS was done first; and if necessary, confirmatory Sanger sequencing. On identification of an index patient with positive germline mutation, they underwent genetic counselling and were advised to contact first degree relatives at risk to undergo genetic counselling and preventive scanning in order to evaluate carrier status. NGS was used for germline mutation only. PCR Sanger sequencing was used for excluding somatic *SDHD* gene mutation as well. Additionally, one patient with cyanotic congenital heart disease also underwent special genetic analysis using single nucleotide peptide (SNP) array to exclude the presence of chromosome 22q11.2 deletion

mutation. In the context of sharing similar research interests for PPGLs, we also performed Whole Exome Sequencing (WES) for certain patients to identify both germline and somatic mutations in collaboration with the National Institutes of Health and National Cancer Institute, Bethesda, USA.

## 3.2 Compliance with ethical standards

Institutional Review Board Statement: All procedures performed in studies involving human subjects were in compliance with the Helsinki declaration and further in accordance with local ethical guidelines of the institutional ethical committees of Charles University, Prague, Czech Republic.

Informed Consent Statement: Additional informed consent was obtained for genetic testing and surgery for all patients according to the hospital regulations, institutional guidelines of Charles University and those defined by the practice codes of the Ministry of Health of Czech Republic.

## 3.3 Subject selection criteria for genetic testing

Inclusion: All patients suspected of or diagnosed with unilateral or multiple HNPGLs between the ages of 18-85 years old; benign or malignant tumors; evaluation of carrier status of relatives of index patients with positive mutation.

Exclusion: Any index patient that did not consent to genetic examination; any first degree relative who did not attend genetic counselling.

## 3.4 Biological materials

Both blood and tumor samples were obtained under appropriate standardized and safe sterile techniques. All samples were transported for genetic analysis to the laboratory using strict precautions and stored in our tissue banks at $-70^0$ C for use in genetic examination.

### 3.4.1 patients' blood samples

All patients who presented to us at the clinic underwent phlebotomy. Genomic DNA was extracted from 10mL of ESTA or ACD-anticoagulated

peripheral blood samples using QIAmp DNA Mini kit (© Qiagen, USA) under standardized conditions.

3.4.2 patients' tumor samples

A 5x5mm tumor block specimen was obtained intraoperatively during surgery and stored in natural state without any storage medium. DNA extraction was done using Puregene Core kit A (© Qiagen, USA).

The quality of all DNA samples was checked with the help of Thermo Scientific™ NanoDrop 2000 and 2000c full-spectrum UV-Vis spectrophotometers (© Thermo Fisher Scientific Inc.)

## 3.5 Protocol for analysis of genetic mutation in HNPGLs

3.5.1 Czech Republic

*3.5.1.1 PCR – Sanger sequencing*

The extracted DNA from peripheral blood samples was analyzed using specific primers for SDHD exons 1-4 (primer sequence available on request). The PCR 25 µl reaction mixture contained 1x PCR buffer (Fermentas), 50 300 ng of genomic DNA as template, 1.5 mM $MgCl_2$ (Fermentas), 25 pmol of each primer, 200 µM of each deoxynucleotide triphosphate (Fermentas) and 1.0 unit of *Taq*DNA polymerase (MBI Fermentas). Amplification conditions included were an initial denaturation at 94Cº for 3 minutes, followed by 35 cycles of 45 seconds at 94 Cº, 45 sec at 60 Cº, 1 minute at 72Cº and final extension step running for 5 minutes at 72 Cº. DNA fragments were sequenced in both forward and backward directions using an automatic fluorescent ABI Prism™ 3130 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer´s instructions. DNA sequence analysis was then done using the Mutation Surveyor® (Carolina Biosystems, Czech Republic).

*3.5.1.2 NGS*

The first steps involved extraction of genomic DNA, quality control of DNA samples and shearing of DNA with end repair of fragments. This was followed by ligation of specific adaptors and ligation of barcodes for multiplex sequencing. Library selection and purification as well as amplification using PCR (bridge PCR

for Illumina) was done in preparation for sequencing. Agilent capture system was used (SSEL XT HS Reagent Kit, Agilent®). Capture-based next-generation DNA sequencing was performed using NextSeq 500 (Illumina®, USA). This is a customized Pheochromocytoma/Paraganglioma gene panel covering the entire coding and selected intronic and promoter regions of 123 genes (Supplementary Table 1) of particular relevance in these tumors. Assembly of sequences and gene annotations was performed. Reads were aligned against the reference genome (GRch38). FinalistDX software was used for Bioinformatics analysis. This method was used for analyzing germline mutations amongst 12 patients with HNPGLs.

### 3.5.2 USA - WES

This analysis was performed in collaboration with the National Institutes of Health (NIH) and National Cancer Institute (NCI), Bethesda, USA. Biological samples were collected from 16 patients and safely transported in dry ice to the National Institutes of Health, Bethesda. Standardized research protocol was followed by the NIH and NCI, Bethesda according to their recommended guidelines. A panel of 20,000 genes were analyzed in the process.

This technique of sequencing consisted two main processes, namely target-enrichment and sequencing. Sample preparation included purification and quality control of DNA samples. The next step was target-enrichment (DNA fragmentation and exome capture). This was performed to select and capture exome from DNA samples. Seventy Exome samples were pooled and sequenced on NovaSeq 6000 S2 (Illumina®, USA) run using Agilent® SureSelect Human All Exon V7 and paired-end sequencing mode. The samples have 100M to 189M pass filter reads, with Q30 above 89%. The samples were mapped and variants were called using Dynamic Read Analysis for GENomics (Dragen; Illumina®, USA). Percent total mapping against reference genome hg38 is about 99% and Uniquely mapped reads are above 12%. Library complexity (i.e. percentage of non-duplicate reads) was determined by measuring the percentage of unique fragments in the mapped reads using MarkDuplicates utility. Percent duplicate reads are between 9% to 85%. There are 52% to 89% of reads mapped on target. Coverage statistics were also measured using Dragen. The mapped sequencing depth coverage over target (after alignment

and marking duplicates) was between 127x to 376x. The mean insert size for these samples was between 161 and 256 bases. More than 64% of the target region have the coverage above 20x.

The *SDHD* germline status was determined for 26 patients using one or a combination of the above techniques. NGS, WES or a combination were used to exclude other *SDHx* and *non-SDHx* gene mutation in a total of 19 patients. PCR and WES were further used in examining somatic mutation in 15 patients.

# 4. RESULTS

## 4.1 Genetic mutations and syndromes related to HNPGLs

On reviewing 92 manuscripts, we found that only 10 out of 15 genes which are directly related to paragangliomas when mutated lead to head and neck paragangliomas (Table 3) (Guha et al., 2019).

Mutations in the *SDHx* genes have the highest affinity for HNPGLs. The *SDHD* gene was found to be the most susceptible gene to be mutated in HNPGLs and is associated with PGL1. This syndrome is most often associated with multiple tumors, a hallmark feature. These are vastly biochemically silent tumors. Age-related penetrance increases with age. The PGL4 syndrome, which is due to a germline mutation in *SDHB* gene, has the highest affinity for developing malignant HNPGLs in comparison to other gene mutations. Such tumors can develop in any PPGL site. *NF1* gene mutation is also considered a high risk for malignant tumors, although much lesser than *SDHB*. PGL3 is a rare disease when compared to PGL1 and PGL4. This *SDHC* gene associated syndrome characteristically shows single HNPGLs and has higher age of diagnosis and low tumor penetrance.

The large study done on the Dutch kindred with the *SDHAF2* mutation has shown age-related tumor penetrance of 100% at 50 years in HNPGLs, and the risk of developing HNPGLs is nearly 100%. Other genes from cluster 1 and 2 related tumors have a much lower affinity for HNPGLs, hence percentage risk, age-related tumor penetrance and prevalence have not been precisely established from our review.

Table 3. Summary of genes with mutations related to HNPGLs *(adapted from Guha et al., 2019)*

| | Cluster 1 | | | | | | | Cluster 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | SDHD | SDHAF2 | SDHC | SDHB | SDHA | VHL | HIF-2α | RET | NF1 | TMEM127 |
| Locus | 11q.23 | 11q13.1 | 1q21 | 1p36.13 | 5p15.33 | 3p25.3 | 2p21-p16 | 10q11.2 | 17q11.2 | 2q11.2 |
| Protein function | Structural subunit of the mitochondrial protein complex II (SDH) | Mitochondrial assembly factor for complex II | Structural subunit of the mitochondrial protein complex II (SDH) | Core subunit of the mitochondrial protein complex II (SDH) | Core subunit of the mitochondrial protein complex II (SDH) | Regulates HIF1a and HIF2a proteasomal degradation | Transcription factor of the bHLH-PAS protein family | Transmembrane tyrosine kinase receptor for extracellular signal molecules of the GDNF family | Inhibits the GTPase HRAS and disrupts the RAS signaling pathway | Probable role in endosomal trafficking and mTOR regulation |
| Syndrome | PGL1 | PGL2 | PGL3 | PGL4 | PGL5 | VHL | Paraganglioma-somatostatinoma-polycythemia | Sipple | NF1 | NA |
| MIM ID | 168000 | 60650 | 605373 | 115310 | 614165 | 193300 | 611783 | 171400 | 162200 | 613903 |
| Inheritance | AD PI | AD PI | AD | AD | AD | AD/Somatic | Somatic | AD PI | AD | AD |
| HNPGL | High | High | Medium | Medium | Low | Very low | Very low | Very low | Very low | Very low |
| Other PGLs | Medium | NA | Low | High | Low | Low | Medium | NA | NA | Variable |
| Multiple PGLs | High | Medium | Low | Medium | NA | Variable | Medium | None | None | None |
| Associated PHEO | Low | None | Variable | Medium | None | High | Low | Medium | Low | High |
| Malignancy risk | Low | NA | Low | High | NA | Low | NA | Low | High | Low |
| Relative Germline mutation Somatic frequency | High | Low | Medium | High | Medium | High | Low | High | Medium | Low |
| | NA | NA | Low | High | NA | High | High | High | Very high | NA |
| Other features | GIST, rarely papillary thyroid cancer | GIST | GIST | GIST rarely renal cell cancer | NA | CNS and eye hemangioblastomas, clear cell renal cancer, inlet cell tumors | Somatostatinoma, polycythemia | Medullary thyroid cancer, pituitary adenoma | Café-au-lait spots, Lisch nodules, fibromatous skin tumors | NA |

AD, autosomal dominant; PI, paternal inheritance; HNPGL, head and neck paraganglioma; PGL, paraganglioma; PHEO, pheochromocytoma; NA, not available; CNS, central nervous system; GIST, gastrointestinal stromal tumor; bHLH-PAS, basic helix-loop-helix-PER-ARNT-SIM; GDNF, glial cell line-derived neurotrophic factor; mTOR, mammalian target of rapamycin

## 4.2 General overview of analyzed patients with HNPGLs

Patient demographics and mode of presentation

A total of 30 patients (36.7% males; 63.3% females) of 34-80 (mean 53) years of age were diagnosed with HNPGLs. Only 5 patients presented below the

age of 40 years old. Twenty-eight patients were of Czech origin, one patient was Hungarian and the other was of Polish origin. Only 2 patients had a positive family history of HNPGLs (Table 4). The most common symptoms were hearing difficulties, tinnitus and painless neck mass. Five patients were diagnosed with incidental HNPGLs on imaging studies and one on intraoperative findings during neck surgery (Table 5).

Characteristics of HNPGLs

Amongst 30 patients, 24 had unilateral and 6 had bilateral/multiple tumors. A total of 42 HNPGLs were found (24 were left-sided, 18 right-sided) (Table 4). According to localization of tumors, we found 11 CBPGLs, 13 JPGLs, 8 TPGLs and 10 VPGLs amongst our cohort of patients.

4.2.1 Unilateral head and neck tumors

*CBPGLs*

A total of 4 patients were diagnosed with single carotid body tumors. All were of Czech origin. Patient no. 4 had a sister with carotid body tumor. The most common symptom reported in patients with carotid body tumors was painless swelling on the affected side. Patient no. 2 was asymptomatic and the tumor was an incidental finding.

*JPGLs*

Eight patients presented with jugular paragangliomas. The youngest patient was of Hungarian origin. All patients complained of hearing difficulties and 3 patients had pulsatory tinnitus.

Patients 7 and 10 presented with varied signs of lower cranial nerve dysfunction (dysphonia, dysphagia and dysarthria), the later had a more severe dysfunction. Patient no. 9 had facial nerve paralysis. All patients had signs of inner ear/cranial nerve VIII dysfunction. Preoperative audiometric analysis amongst this group of patients showed three had mixed hearing loss, 3 were diagnosed with sensorineural hearing loss and 1 had conductive hearing loss (Table 5).

Table 4. Summary of analyzed patients with HNPGLs *(Guha & Chovanec, 2021)*

| Pt. | Age (yrs.) | Gender | Medical History | F/H of PPGLs | Tumor(s) | | Type of Treatment |
|---|---|---|---|---|---|---|---|
| | | | | | Laterality | Classification | |
| 1 | 47 | M | Asthma | - | R | Shamblin II | Surgery |
| 2 | 62 | M | Hypertension | - | L | Shamblin II | Surgery |
| 3 | 64 | M | Pangastritis | - | R | Shamblin II | Surgery |
| 4 | 76 | F | - | + | R | Shamblin I | W+S |
| 5 | 37 | F | Asthma, hypertension, renal angiomyolipoma | - | L | Fisch C3 Di2 | Surgery |
| 6 | 37 | F | - | - | L | Fisch C3 | Surgery |
| 7 | 43 | F | Hypertension, hydrocephalus | - | L | Fisch C3 Di3 | Surgery |
| 8 | 54 | F | Hypertension, diabetes mellitus | - | R | Fisch C3Di1 | Surgery |
| 9 | 60 | F | Hypertension | - | L | Fisch C3 De2 | Surgery |
| 10 | 64 | M | Diabetes mellitus | - | L | Fisch C3 | Surgery |
| 11 | 69 | F | Diabetes mellitus, Leiden mutation | - | R | Fisch C1 | Surgery |
| 12 | 80 | F | Hypertension | - | L | Fisch C3 Di3 | W+S |
| 13 | 42 | M | Hypertension, Diabetes mellitus | - | L | Fisch B3 | Surgery |
| 14 | 48 | F | Hypertension | - | L | Fisch A1 | Surgery |
| 15 | 48 | F | - | - | R | Fisch B2 | Surgery |
| 16 | 55 | F | Hypothyroidism, Duodenal ulcer | - | L | Fisch B2 | Surgery |
| 17 | 57 | M | - | - | L | Fisch C2 Di1 | W+S |
| 18 | 67 | F | - | - | R | Fisch C1 | Surgery |
| 19 | 71 | F | - | - | L | Fisch C1 | W+S |
| 20 | 39 | F | Asthma | - | L | Fisch A | Surgery |
| 21 | 44 | F | Hypothyroidism | - | R | Fisch A | Surgery |
| 22 | 46 | M | - | - | R | Fisch A | Surgery |
| 23 | 51 | F | - | - | R | Fisch A | Surgery |
| 24 | 51 | F | Hypertension, Migraine | - | L | Fisch A | Surgery |
| | | | | | | | |
| 25 | 34 | M | Tetralogy of Fallot | - | B | L: Shamblin III, R: Shamblin II | W+S |
| 26 | 36 | M | Paranoid schizophrenia | - | B | Shamblin III | Treatment |
| | | | | | B | Fisch C | declined |
| | | | | | R | Fisch C4 Di2 | Deceased |
| 27 | 43 | F | Spontaneous abortion | + | R | Shamblin II | Surgery |
| | | | | | L | Fisch A | W+S |
| 28 | 47 | M | - | - | L | Fisch B | W+S |
| | | | | | L | Fisch C1 | W+S |
| 29 | 51 | M | Hypertension | - | B | Shamblin II | Surgery |
| | | | | | B | Fisch C1 | Surgery |
| | | | | | L | Fisch A1 | W+S |
| 30 | 57 | F | Hypertension, Hyperlipidemia | - | R | Fisch A | Surgery |
| | | | | | L | Fisch C1 | W+S |

CBPGL
JPGL
TPGL
VPGL

B – Bilateral; F – Female; L – Left; M – Male; R – Right; W+S – Wait and Scan

*TPGLs*

Out of 7 patients diagnosed with tympanic tumors, one was a second recurrence. All patients complained of hearing difficulty and objectively had conductive hearing loss. Only 3 patients had pulsatory tinnitus (Table 5). Patient no. 15 also had an incidental finding of frontoparietal meningioma on MRI. All patients had conductive hearing loss.

Table 5. Mode of presentation amongst patients with HNPGLs
*(Guha & Chovanec, 2021)*

| | | **Number of patients** *[Total (N = 30)]* n (%) |
|---|---|---|
| **Demographic profile** | | |
| Gender | *Males* | 11 (36.7%) |
| | *Females* | 19 (63.3%) |
| Age at presentation below 40 years | | 5 (16.7%) |
| Medical history of hypertension | | 11 (36.7%) |
| Patients with negative family history | | 28 (93.3%) |
| **Clinical features** | | |
| Asymptomatic | | 2 (6.7%) |
| Painless neck mass | | 10 (33.3%) |
| Pulsation in the neck | | 1 (3.3%) |
| Dysphonia/hoarseness of voice | | 3 (10%) |
| Dysphagia | | 3 (10%) |
| Dysarthria | | 2 (6.7%) |
| Facial nerve palsy | | 2 (6.7%) |
| Restrictive tongue movement | | 1 (3.3%) |
| Hearing difficulty | | 16 (53.3%) |
| Pulsatile tinnitus | | 8 (26.7%) |
| Pressure in the ear | | 1 (3.3%) |
| Otorrhagia | | 1 (3.3%) |
| **Imaging studies** | | |
| Presence of HNPGLs as incidentalomas | | 5 (16.7%) |
| Presence of HNPGLs | *Solitary* | 24 (80%) |
| | *Multiple* | 6 (20%) |
| Presence of PGLs below the neck | | 3 (10%) |

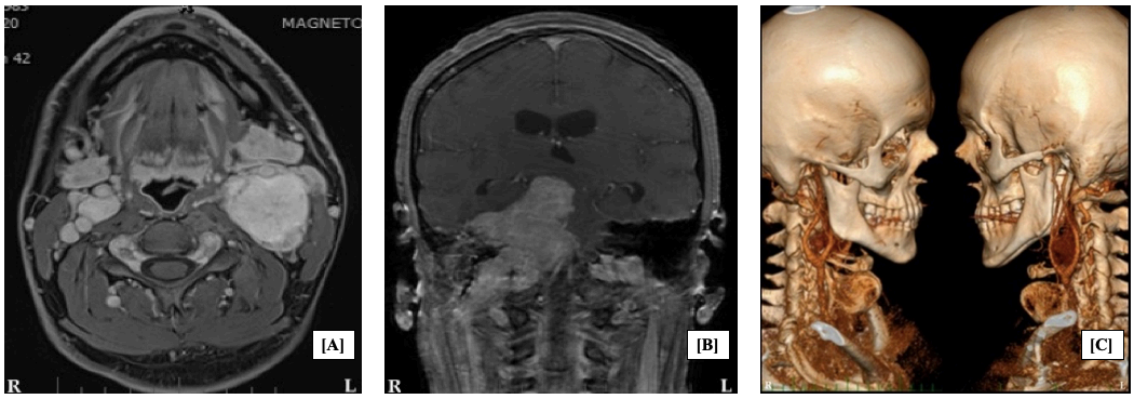
*VPGLs*

Five patients were diagnosed with unilateral vagal tumors. One patient had an incidentaloma, diagnosed on follow up ultrasound scan of the neck for hypothyroidism and another was diagnosed intraoperatively as suspected metastasis of thyroid gland cancer. All patients presented with painless lump in the neck.

4.2.2 Bilateral or multiple HNPGLs

Six patients were diagnosed with 18 multiple benign HNPGLs (Table 4). Bilateral CBPGLs were seen in 3 patients (Figure 4).

Figure 4. Bilateral CBTs in patients with multiple HNPGLs *(adapted from Guha et al., 2021)*

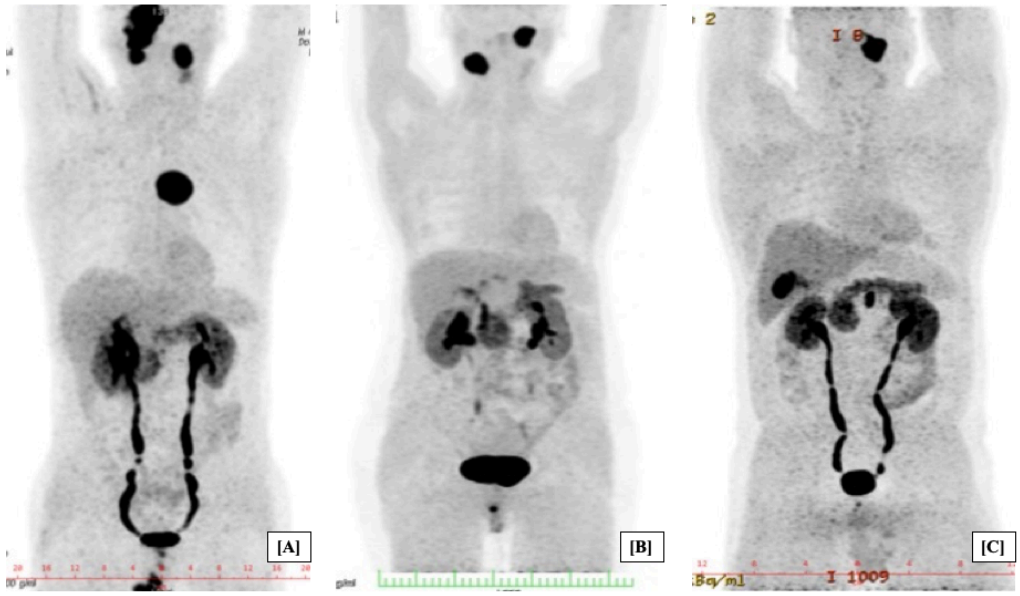



[A] MRI of the neck (axial view) in Patient no. 25 with congenital heart disease and absence of germline mutation; [B] MRI of the head and neck (coronal view) in Patient no. 26 with advanced disease and *SDHD* mutation; [C] 3D reconstruction of CT angiography neck (lateral views) in Patient no. 29 with absence of germline mutation.

Patient no. 25 had bilateral CBPGLs (Shamblin III on the left, Shamblin II on the right) and a rare form of Tetralogy of Fallot, a cyanotic congenital heart disease. This consisted of pulmonary atresia with ventricular septal defect, which was left unrepaired leading to cardiopulmonary complications and hypoxemia. Patient no. 26 had very advanced disease and confirmed dysfunction of VII-XII cranial nerves. The 51-year old patient in our series was also diagnosed incidentally with 5 HNPGLs as he presented with intractable otorrhagia. Multifocal PGLs were seen in 3 patients (Figure 5).

Only the patient of Polish origin (patient no. 27) had a positive history of VPGL on her father's side of the family. Patient no. 28 had incidental findings of left sided vagal and jugular tumors, which have been stable with minimal progress over the last 10 years. The last patient developed a JPGL after 3 years of follow up.

Figure 5. Whole body imaging using 18F-FDOPA PET/CT demonstrating multifocal paragangliomas *(adapted from Guha et al., 2021)*




[A] Bilateral CBPGLs, VPGLs and unilateral JPGL with mediastinal sPGL is seen in patient no. 26; [B] Unilateral CBPGL, VPGL and retroperitoneal sPGL is seen in patient no. 27; [C] Unilateral VPGL, JPGL and retroperitoneal sPGL is seen in patient no. 28.

## 4.3 Genetic sequencing results

### 4.3.1 Solitary tumors

Out of 24 patients with solitary HNPGLs, four patients did not undergo genetic examination (Table 6). The *SDHD* germline mutation was not found in any of the patients.

i. Carotid body tumors: three patients were examined; one did not consent. No germline mutation was found, including the 76-year old with CBPGL who also had a sister with the same tumor. However, the *IDH2* somatic mutation was found in one of the patients.

ii. Jugular paragangliomas: all the patients underwent analysis of germline *SDHD* mutation using a single or combination method. NGS and or WES was performed amongst 6 out of 8 patients with such tumors. The *SDHB* germline mutation was identified on WES in a 37-year old female Czech patient. Out of 6 patients' tumors analyzed for somatic mutation, *SDHB* was detected in a 64-year old male patient.

Table 6. Analysis of genetic mutation amongst patients with solitary tumors

| Pt. | Age (yrs) | F/H | Tumor Site | GERMLINE PCR *SDHD* | GERMLINE NGS | GERMLINE WES (USA) | SOMATIC PCR *SDHD* | SOMATIC WES (USA) |
|---|---|---|---|---|---|---|---|---|
| 1 | 47 | - | CBPGL (R) | Negative | No variant identified | No variant identified | Negative | *IDH2:* c.515G>A (p.Arg172Lys) |
| 2 | 62 | - | CBPGL (L) | X | X | X | 0 | 0 |
| 3 | 64 | - | CBPGL (R) | X | No variant identified | X | 0 | 0 |
| 4 | 76 | + | CBPGL (R) | Negative | X | X | N/A | N/A |
| 5 | 37 | - | JPGL (L) | Negative | No variant identified | No variant identified | Negative | No variant identified |
| 6 | 37 | - | JPGL (L) | Negative | X | *SDHB:* c.689G>T (p.R230L) | Negative | No variant identified |
| 7 | 43 | - | JPGL (L) | Negative | No variant identified | X | 0 | 0 |
| 8 | 54 | - | JPGL (R) | X | No variant identified | No variant identified | Negative | No variant identified |
| 9 | 60 | - | JPGL (L) | Negative | X | No variant identified | Negative | No variant identified |
| 10 | 64 | - | JPGL (L) | Negative | X | No variant identified | Negative | *SDHB:* c.600G>C (p.Trp200Cys) |
| 11 | 69 | - | JPGL (R) | X | No variant identified | No variant identified | Negative | No variant identified |
| 12 | 80 | - | JPGL (L) | Negative | X | X | N/A | N/A |
| 13 | 42 | - | TPGL (L) | Negative | X | X | 0 | 0 |
| 14 | 48 | - | TPGL (L) | Negative | X | No variant identified | Negative | No variant identified |
| 15 | 48 | - | TPGL (R) | Negative | X | No variant identified | Negative | No variant identified |
| 16 | 55 | - | TPGL (L) | Negative | X | X | 0 | 0 |
| 17 | 57 | - | TPGL (L) | Negative | X | X | N/A | N/A |
| 18 | 67 | - | TPGL (R) | Negative | X | X | 0 | 0 |
| 19 | 71 | - | TPGL (L) | X | X | X | N/A | N/A |
| 20 | 39 | - | VPGL (L) | Negative | X | X | 0 | 0 |
| 21 | 44 | - | VPGL (R) | X | *SDHB*: c.689g>a (p.arg230His) | X | Negative | No variant identified |
| 22 | 46 | - | VPGL (R) | X | X | X | Negative | No variant identified |
| 23 | 51 | - | VPGL (R) | X | X | *SDHB:* c.252C>A (p.D84E) | Negative | No variant identified |
| 24 | 51 | - | VPGL (L) | X | X | X | Negative | No variant identified |

X – Examination not performed; 0 – Tumor sample unavailable; N/A – Not applicable

iii. Tympanic paragangliomas: six patients tested negative for PCR sequencing. NGS examination was not carried out for any of the patients. Only 2 patients underwent WES (germline and somatic) without identification of mutations. One patient did not undergo genetic examination.

iv. Vagal paragangliomas: patient no. 21 tested positive for germline *SDHB* mutation on NGS examination whilst patient no. 23 had another germline *SDHB*

mutation on WES examination. Four out of five patients underwent PCR *SDHD* and WES to exclude somatic mutations, none were discovered.

4.3.2 Bilateral or multiple tumors

Amongst those with multiple tumors, *SDHD* germline mutation was found in 3 patients. Patients no. 26 and 30 were positive on NGS examination, whilst the Polish patient was diagnosed with *SDHD* on PCR sanger sequencing. *SDHB* mutation was detected in patient no. 28. The other 2 cases were negative on NGS (Table 7). Analysis of somatic examination on tumor samples of 2 patients revealed no mutation. Furthermore, the *RET* mutation was found in the tumor sample extracted from the retroperitoneal sPGL of patient no. 26. More interestingly, SNP array sequencing excluded the presence of the chromosome 22q11.2 deletion mutation in patient no. 25 with the congenital cyanotic heart disease and bilateral carotid body tumor. No abnormal variant was found on PCR, NGS and WES.

Table 7. Comparative analysis of patients with multiple tumors

| Pt. | Age (yrs.) | Gender | F/H | Plasma MET nmol/l | Plasma NMET nmol/l | CgA ng/ml | Germline mutation | Syndrome | Somatic mutation | Type and localization of PGLs |
|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 34 | M | - | 0.063 | 0.308 | 32.7 | NGS + WES: No variant Identified | - | N/A | Carotid (B) |
| 26 | 36 | M | - | 0.012 | 0.186 | 231.4 | NGS: *SDHD:* c.1A>G (p.Met1Val) | PGL1 | N/A | Carotid (B)<br>Vagal (B)<br>Jugular (R)<br>Anterior Mediastinum |
| 27 | 43 | F | + | 0.171 | 0.342 | 224.7 | PCR + WES: *SDHD:* c.112C>T,p.R38 | PGL1 | PCR + WES: No variant Identified | Carotid (R)<br>Vagal (L)<br>Retroperitoneal |
| 28 | 47 | M | - | 0.102 | 1.306 | 72.6 | NGS: *SDHB:* c.287G>A (p.Gly96Asp) | PGL4 | N/A | Vagal (L)<br>Jugular (L)<br>Retroperitoneal |
| 29 | 51 | M | - | 0.186 | 0.231 | 22.5 | NGS + WES: No variant Identified | - | PCR + WES: No variant identified | Carotid (B)<br>Jugular (B)<br>Tympanic (L) |
| 30 | 57 | F | - | 0.031 | 0.285 | 78.2 | NGS: *SDHD:* c.53-2A>G | PGL1 | 0 | Vagal (R)<br>Jugular (L) |

MET – Metanephrine; NMET – Normetanephrine; CgA – Chromogranin A; N/A – Not applicable

## 4.4 Summary of other results

### 4.4.1 Biochemical tests

Plasma metanephrine (0.140 - 0.540 nmol/l) and normetanephrine (0.130 – 0.790 nmol/l) levels were within physiological limits for all patients, hence all tumors were of non-secretory nature. All patients had normal levels of the tumor marker Chromogranin A (0-85ng/ml), except for patients no. 7 (156.5 ng/ml), 26 (231.4 ng/ml) and 27 (224.7 ng/ml) (Table 7).

### 4.4.2 Treatment modality and outcome

After completion of all examination, patients were either allocated to surgery or 'wait and scan' approach. A total of 23 patients (20 with unilateral single tumors, 3 with multiple HNPGLs) underwent surgery. Tumor embolization was carried out as a preoperative measure for patients undergoing surgery as per recommended standards of safe practice.

No complications were encountered in any of the patients following embolization. However, in two patients with extensive jugular paragangliomas, deterioration of cranial nerve status was seen following intervention. Patient no. 6 had lower cranial nerve as well as facial nerve dysfunction and patient no. 10 had VIII-XII nerve palsy. In both of these patients, primary intraoperative findings showed encasement of affected cranial nerves with the tumor.

Tumor excision and selective neck dissection (SND) were carried out for all patients with CBPGLs, JPGLs and VPGLs. Amongst those with CBPGLs, perioperative and postoperative complications such as stroke and hemorrhage were not seen. Five patients with TPGLs had extirpation of the paraganglioma; patient no.18 additionally underwent SND. Status of cranial nerve function in patients who underwent surgery is demonstrated in Figure 6. Patients with multiple tumors underwent surgery in a step-wise manner.

Figure 6. Status of cranial nerve function in patients who underwent surgery *(adapted from Guha & Chovanec, 2021)*

| | Time of diagnosis /Preoperatively | | | | | | Postoperatively | | | | | | On last follow-up | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pt.** | VII | VIII | IX | X | XI | XII | VII | VIII | IX | X | XI | XII | VII | VIII | IX | X | XI | XII |
| 1 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 2 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 3 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | | | | | | | | | | |
| 5 | HB1 | M | 0 | 0 | 0 | 0 | HB6 (R) | L | 0 | 0 | 0 | 0 | HB4 | L | 0 | 0 | 0 | 0 |
| 6 | HB6 | S | + | + | 0 | 0 | HB6 (R) | L | + | + | 0 | 0 | HB3 | L | 0 | 0 | 0 | 0 |
| 7 | HB1 | M | + | + | 0 | 0 | HB3 | L | 0 | 0 | 0 | 0 | HB1 | L | 0 | 0 | 0 | 0 |
| 8 | HB1 | S | 0 | 0 | 0 | 0 | HB1* to 2** | L | + | + | + | 0 | HB1 | L | 0 | 0 | + | 0 |
| 9 | HB6 | M | 0 | 0 | 0 | 0 | HB6 | L | 0 | 0 | 0 | 0 | HB6 | L | 0 | 0 | 0 | 0 |
| 10 | HB1 | S | + | + | + | + | HB1* to 4** | L | + | + | + | + | HB1 | L | + | + | + | + |
| 11 | HB3 | C | 0 | 0 | 0 | 0 | HB4 | C | + | + | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | | | | | | | | | | |
| 13 | HB1 | C | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 14 | HB1 | C | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 15 | HB1 | C | 0 | 0 | 0 | 0 | HB1* to 3** | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 16 | HB1 | C | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 18 | HB1 | C | 0 | 0 | 0 | 0 | HB3* to 5** | 0 | 0 | 0 | 0 | 0 | HB3 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | | | | | | | | | | |
| 20 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | + | + | 0 | HB1 | 0 | 0 | $+_c$ | 0 | 0 |
| 21 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | + | 0 | 0 | HB1 | 0 | 0 | $+_c$ | 0 | 0 |
| 22 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | + | 0 | 0 | HB1 | 0 | 0 | $+_c$ | 0 | 0 |
| 23 | HB1 | 0 | 0 | 0 | 0 | + | HB1 | 0 | 0 | + | 0 | + | HB1 | 0 | 0 | $+_c$ | 0 | 0 |
| 24 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | + | 0 | + | HB1 | 0 | 0 | $+_c$ | 0 | 0 |
| | | | | | | | | | | | | | | | | | | |
| 27 | HB1 | 0 | 0 | 0 | 0 | 0 | HB2 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 29 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | 0 | 0 | 0 |
| 30 | HB1 | 0 | 0 | 0 | 0 | 0 | HB1 | 0 | 0 | + | 0 | 0 | HB1 | 0 | 0 | $+_c$ | 0 | 0 |

Red = CBPGL; Yellow = JPGL; Green = TPGL; Blue = VPGL; Purple = Multiple PGLs; HB: House-Brackmann Scale; M: Mixed Hearing Loss; C: Conductive Hearing Loss; S: Sensorineural Hearing Loss; R: Reconstructed; L: Labyrinthectomy leading to deafness; *: early postoperative function; **: 10week postoperative function; $+_c$ compensated nerve dysfunction).

Patient no. 25 was deemed medically unfit due to the cardiopulmonary condition related to the advanced congenital cardiovascular disease. The patient with multifocal paragangliomas and *SDHB* mutation declined surgery and opted for 'wait and scan' approach. So, both patients were closely monitored. One patient was kept under observation, but, the disease had already progressed considerably. He died from respiratory complications of very advanced disease due to bilateral lower cranial nerve palsies. The rest of them were allocated to 'wait and scan' method. Radiotherapy was not used amongst our cohort of patients as first line modality of treatment.

Annual follow up for all patients includes MRI for anatomical evaluation. Functional imaging using PET/CT is currently performed every 3 years for patients with proven germline mutation and or multiple tumors. Status of cranial nerve

function on last follow up is given in Figure 6. Only the last patient in our series with *SDHD* mutation was diagnosed with a JPGL 3 years later on follow up imaging. The patients with *SDHB* mutations and the only one with the malignant tumor had no further progression of tumors or development.

4.4.3 Histopathology results

On histopathology examination, paragangliomas were confirmed on the basis of the classic 'Zellballen' pattern. All surgically removed tumors except one were confirmed as benign with no metastatic disease on histopathology examination. Tumor metastasis in the otherwise non-enlarged lymph node was found only in patient no. 8 with a solitary jugular PGL.

# 5. DISCUSSION

The initial study done in Czech Republic reiterated the lack as well as the importance of data related to HNPGLs. Only 3 patients with HNPGLs were found amongst 64 patients analyzed in this long-term study. All the 3 patients were younger than 40 years. The youngest patient was a 23-year old male with positive family history and neck metastasis including carotid body infiltration from the mediastinum. Interestingly, 1 of 3 patients with HNPGLs had *SDHD* germline mutation, the most common mutation found in these patients (Vosecka et al., 2017). We also studied the frequency of BRAF mutations, which varies widely amongst human cancers. There is also a controversial view whether this mutation contributes to PPGLs or not (Lenders et al., 2014; Martucci & Pacak, 2014). BRAF mutation is a very good treatment target; the presence of this mutation in some of these tumors could result in the use of B-Raf inhibitors for metastatic forms of the disease for which effective treatment has not been established thus far. The BRAF V600E mutation was not found in any of analyzed patients' samples (Vosecka et al., 2017). Only one study detected the V600E *BRAF* mutation in 1.2% (1/85) of these tumors (Luchetti et al., 2015). Till date, 427 PPGLs were investigated for the presence of a *BRAF* mutation, a gene mutation found only in 1 of these tumors, suggesting that the *BRAF* V600E mutation is an extremely rare genetic event seen in

pheochromocytomas and paragangliomas (Luchetti et al., 2015). In present times, it would not serve as a target for new treatment options of metastatic PPGLs.

Our systematic review demonstrates that considerable progress has been made in the genetics of paragangliomas over the last few years. Before the year 2000, only one genetically determined form of the disease was thought to exist, but it has now been shown that about 30% of paragangliomas are genetically determined due to the presence of mutation in one of the 15 susceptibility genes identified to date. We found that only 10 of these genes have been related to HNPGLs (Guha et al., 2019). The clinical presentation including location of tumors, multifocality as well as malignancy varies vastly with the gene mutated. The findings of our review also reiterate that *SDHD* mutation is the most commonly mutated gene in HNPGLs and has a hallmark of multiple tumors (Guha et al., 2019, 2021). This is followed by *SDHB* and *SDHC*. *SDHB*-related PGLs are usually large, solitary and show a high frequency for malignancy. These mutations are most frequently implicated in the pathogenesis of PPGLs (including HNPGLs) in both adults and children (Guha et al., 2019; Karasek et al., 2013). Malignancy with respect to hereditary background can be further demonstrated in *NF1*, *VHL*, *RET* and *TMEM127* gene mutations (Fliedner et al., 2010; Guha et al., 2019). Multiple tumors, positive family history, age at diagnosis less than 40 years, presence of carotid body tumor as well as bilateral presentation should be considered hallmarks of germline mutations in HNPGLs (Burnichon et al., 2009; Fakhry et al., 2008; King & Pacak, 2014; Neumann et al., 2009; Piccini et al., 2012). In addition, 30% of HNPGLs which are considered sporadic in the absence of positive family history, should be evaluated as occult familial cases, an important concept. In summary, germline mutations in *SDHAF2* mutations are associated with HNPGLs. *SDHA* and *SDHC* are associated with HNPGLs and sympathetic paragangliomas. *SDHD* and *SDHB* mutations are associated with HNPGLs, sPGLs and PHEOs, whilst *TMEM127* and *MAX* are more common with PHEOs (Guha et al., 2019).

The cohort of patients we studied showed higher female predominance (females: males = 1.7:1), but this was less than the expected for females (Boedeker, 2011). It has also been well established that amongst all HNPGLs, carotid body tumors are the most common type (60% and more) (Patetsios et al., 2002), followed

by jugulotympanic (<35-40%) and vagal (<5%) paragangliomas. However, in our cohort of patients with HNPGLs, we showed a different distribution pattern. Amongst 42 tumors found, 31% were JPGLs, followed by CBPGLs (26.2%), VPGLs (23.8%) and TPGLs (19%) (Guha & Chovanec, 2021). These findings are comparative to a study done in Germany with a large series of patients, where they showed JPGLs (39%), CBPGLs (30.5%), TPGLs (14.3%) and VPGLs (11.7%) (Papaspyrou et al., 2012). Although, other forms of head and neck paragangliomas were absent in our case series, we found other PGLs in 3 of our patients. Additionally, 2 out of 30 patients reported here had positive family history, this includes 16.7% of our patients with multiple HNPGLs. A similar study showed 20 out of 79 patients with multiple tumors and only 4 with positive family history (Piccini et al., 2012). The most common symptoms associated with our group of patients were hearing loss (53.3%) followed by painless neck mass (33.3%) and pulsatile tinnitus (26.7%). Only 5 (16.7% of all) patients had different cranial nerve deficits. Interestingly, cranial nerve deficits were absent in patients with Shamblin II/III CBPGLs. This was relatively different in comparison to a study done on HNPGLs by the Mayo Clinic, where only 5% reported decreased hearing (Erickson et al., 2001). Five of our patients had incidental findings of HNPGLs, which is not an uncommon phenomenon on ultrasound of the neck indicated for other neck pathology especially thyroid disease (Guha et al., 2019; Guha & Chovanec, 2021).

Researchers worldwide reported that head and neck paragangliomas (solitary or multiple) are strong predictors of *SDHD* mutation (Table 8) even in small cohort of patients (Astuti et al., 2003; Badenhop, 2004; Benn et al., 2006; Fakhry et al., 2008; Lima et al., 2007; Pandit et al., 2016). Furthermore, there is an emerging evidence that the incidence is relatively high in Europe (Dannenberg et al., 2002; Fakhry et al., 2008; Hensen et al., 2011; Lima et al., 2007; Neumann et al., 2004; Papaspyrou et al., 2012; Piccini et al., 2012; Schiavi et al., 2005). This also holds true for apparently non-familial cases, especially in multiple tumors (Astuti et al., 2003; Fakhry et al., 2008; Hensen et al., 2011; Piccini et al., 2012), as demonstrated in our comparative analysis of multiple HNPGLs (Guha et al., 2021). Netherlands established the highest absolute prevalence of PGL1 (Dannenberg et al., 2002; Hensen et al., 2011). The *SDHD* mutation was surprisingly found in only

3 patients (two of Czech ethnicity) in our series. Two out of these three patients had CBPGLs, one had bilateral tumors. Both Czech patients with *SDHD* mutation had negative family history. Otherwise two other patients also had bilateral CBPGLs but were negative for a germline mutation. However, presence of carotid body tumor as well as bilateral presentation should also be considered as germline-related HNPGLs especially with *SDHD* mutation (Neumann et al., 2009), which supports our findings. These tumors are usually of non-hereditary form in about 60% of the cases (Guha et al., 2019; Robertson et al., 2019), the rest should be considered as hereditary syndromes in the existence of clinical predictors (Burnichon et al., 2009; Neumann et al., 2009; Smith et al., 2017). Only 16.7% of our patients' group were young patients (less than 40 years of age), four were of Czech ethnicity (1 had *SDHD* mutation) and 1 of Hungarian origin. Amongst these young patients, the patient with *SDHD* mutation had multiple tumors (including bilateral CBPGLs). A large number of studies also showed that the percentage of germline *SDHD* mutations in positive family history could be as high as up to 100% (Table 8). In our series, only 1 patient with positive family history had *SDHD* mutation but she was of Polish origin, that is 50% of all with known family history. As discussed previously, maternally derived *SDHD* mutation carriers will still pass the mutation to their offspring in 50% of cases, hence PGL1 can seem to skip generations. This partly explains the high occurrence of *SDHD* germline mutations in 'occult familial' cases. However, only 8.3% of our patients could be considered as *SDHD*-related occult familial cases, thus disproving our hypothesis. Paternal transmission is also associated with incomplete penetrance (Taïeb et al., 2014).

The *SDHD* germline mutation c.1A>G (p.Met1Val) found in our patient, with bilateral CBPGLs, bilateral VPGLs and right-sided JPGL, was a missense pathogenic mutation (Guha et al., 2021) . Interestingly, this type of mutation was also reported in Germany in nonfamilial cases associated with carotid, vagal, jugular and tympanic PGLs (Riemann et al., 2004) and later in a family of PGLs in China (Wang et al., 2012). The next *SDHD* missense mutation c.112C>T(p.R38) in the Polish patient with positive family history (Guha et al., 2021) was identified amongst familial cases in USA (Baysal, 2002) and in an unrelated French index case with non-functional HNPGL (Neumann et al., 2004). Other large European

studies reported the same mutation in HNPGLs (Burnichon et al., 2009; Erlic et al., 2009). The last *SDHD* splicing mutation c.53-2A>G in the eldest patient (Guha et al., 2021) was sparingly reported by a large study in 2009 (Neumann et al., 2009) and subsequently in Spanish patients (Hermsen et al., 2010). Furthermore, comparative analysis also proves most studies from Europe showed a high rate of *SDHD* mutations in comparison to other *SDHx* mutations in the pathogenesis of HNPGLs (Table 8).

Table 8. Worldwide distribution of *SDHD* mutations in HNPGLs

| Country of study | Study Reference | Study Timeline | No. of cases with HNPGLs | % *SDHD* mutations | % *SDHD* mutations with +ve family history | % *SDHD* /Total *SDHx* mutations |
|---|---|---|---|---|---|---|
| **Single center** | | | | | | |
| **Australia** | *Badenhop et al. 2004* | 1991-2001 | 34 | 32.3 | 80 | 78.6 |
| **Czech Republic** | *Guha et al. 2022* | 2016-2021 | 26 | 11.5 | 50 | N/A |
| **France** | *Fakhry at al. 2008* | 1994-2007 | 23 | 26 | 100 | 75 |
| **Germany** | *Papaspyrou et al. 2011* | 1989-2010 | 175 | 12.5 | 100 | 64.7 |
| **India** | *Pandit et al. 2016* | Unspecified | 10 | 10 | N/A | 100 |
| **Italy** | *Piccini et al. 2012* | 2003-2011 | 79 | 80.5 | 100 | 85 |
| **Netherlands** | *Hensen et al. 2011*<br>*Dannenberg et al. 2002*<br>*Taschner et al. 2001* | 1950-2009<br>1987-1999<br>Unspecified | 236<br>57<br>87 | 83<br>56.1<br>59.7 | 99.5<br>100<br>96.9 | 91.5<br>100<br>N/A |
| **Spain** | *Lima et al. 2007* | 1981-2005 | 40 | 12.5 | 79 | 41.7 |
| **United Kingdom** | *Astuti et al. 2003* | 1990-1999 | 34 | 11.8 | 100 | 100 |
| **Multicenter** | | | | | | |
| **Australia, Canada, France, Germany, New Zealand, United Kingdom and United States** | *Benn et al. 2006* | 2003-2004 | 27 | 89 | 69 | 89 |
| **Germany, Poland** | *Neumann et al. 2004* | 2000-2004 | 83 | 79 | N/A | 73 |
| **Italy, Finland, France, Poland, Spain and Switzerland** | *Schiavi et al. 2005* | 2001-2004 | 121 | 16.5 | 66.6 | 60.6 |

We could not substantiate this in our group of patients (Guha et al., 2021; Guha & Chovanec, 2021), where only 11.5% of examined patients had this mutation. Furthermore, if we studied Czech patients only, then the frequency lowers to 8%. We found one Eastern European study that had a similar deduction like ours. This study from Russia that examined 91 patients of Slavic origin with HNPGLs, found 9 patients with 6 variant *SDHD* mutations. They concluded that *SDHD* mutations were less common in comparison to other European countries (Shulskaya

et al., 2018). Although we present a smaller cohort of patients but if we compare rarity of HNPGLs versus timeline, then our number of cases becomes significant. There is a discrepancy that arose between the expected and actual outcome in terms of *SDHD* mutation. A number of suggestions or theories may be offered to explain this divergence. We could contemplate that patients may be unaware of their family history or their family members remained asymptomatic and therefore undiagnosed. It has been proposed that the probability of ascertaining a mutation decreases to 40% in patients without a family history. Patients with germline mutations are not only at risk of developing multiple tumors but may also transmit the mutation to the next generation (Taschner et al., 2001).

The only other germline mutation found in our patients was linked to the *SDHB* gene. Risk of malignancy with *SDHB* mutation is higher (Neumann et al., 2009), however in our series, amongst four patients with *SDHB* mutation, malignancy could not be proved. Three patients with solitary tumors were both clinically and histopathologically confirmed as benign. The patient with multiple tumors was difficult to precisely assess for malignity, since clinical and radiographic findings did not support metastasis and he did not undergo surgery. On the other hand, the patient who was diagnosed with malignancy, had no family history and was also negative for any germline mutation. The 47-year old male patient with multiple PGLs was diagnosed with PGL4 due to the *SDHB* missense mutation c.287G>A (p.Gly96Asp) (Guha et al., 2021), a mutation profile that was also reported in a patient with c.18A>C (p.Ala6Ala) and a c.201–36T>G polymorphism and malignant catecholamine producing paragangliomas (Brouwers et al., 2006) as well 2 pediatric patients with functional tumors (Jochmanova et al., 2020). Two patients with solitary VPGLs were found to have the mutation. The SDHB:c.689g>a (p.arg230His) missense mutation was found in a female patient of 44 years with solitary VPGL, which was reported amongst a series from USA, that studied patients with malignant PGLs (Brouwers et al., 2006). The second patient with VPGL was also a female. Here we determined the presence of SDHB:c.252C>A(p.D84E). This was a missense mutation of likely pathogenic clinical significance, basically of benign nature, associated with PGL4 syndrome and pheochromocytoma, as reported by the gene database, National Center for

Biotechnology Information, National Library of Medicine. But definitive cases with HNPGLs were not found. The last patient in the series of patients with germline SDHB mutation was a 37-year old female patient with solitary JPGL. This was SDHB:c.689G>T(p.R230L), a gene mutation reported from one study in Poland (Michałowska et al., 2016). Our study showed a higher frequency of SDHB mutation, similar to a study done in Belgium (Persu et al., 2008). Finally, it should be mentioned that we found 22% *SDHB*-related 'occult familial' cases in 18 examined patients. Although it is more common with *SDHD*, but it can also be seen in other *SDHx* genes too. We were unable to test relatives of index patients, due to lack of consent and cooperation, hence this was one of the pitfalls of the study.

Out of 23 patients who underwent surgery, tumor samples from 15 patients were examined for somatic mutation. Only 2 male patients with solitary tumors were positive for this. The Isocitrate Dehydrogenase 2 (IDH2) mutation was found in a 47-year old patient with a carotid body tumor. This is a very rarely reported mutation in patients with PPGLs (Lang et al., 2021), and as such not even a major marker for sporadic tumors (Yao et al., 2010). But of significant interest, is that, the variant IDH2:c.515G>A(p.Arg172Lys) found in our patient is mostly found in myelodysplastic syndromes (Petrova et al., 2018) or aggressive cancers (e.g. glioblastoma or hepatocellular carcinoma)(Chang et al., 2016). This patient has not had any recurrence and was diagnosed with a benign tumor. The other somatic mutation was seen in a 64-year old patient with jugular tumor. This was a form of *SDHB* mutation. This mutation, SDHB:c:600G>C(p.Trp.200Cys) has been more commonly reported with PPGLs (Jochmanova et al., 2017). And similarly, despite being of SDHB in origin, the tumor was of benign character. Although somatic mutations do not alter the definitive management of such tumors these influence the timings of follow-ups for these patients.

Our study shows that an integral part of management is early and accurate diagnosis of patients suspected of head and neck paragangliomas. The choice of investigative techniques used in our patients is the current standard approach used in centers where patients with HNPGLs are managed (Nölting et al., 2019; Offergeld et al., 2012; Smith et al., 2017). Accordingly, we suggest that management of HNPGLs (including multiple HNPGLs) should be approached in 4

ways (Guha et al., 2021) and can be categorized as (1) preventive (2) intermediate and (3) definitive and (4) innovative.

## 5.1 Management of HNPGLs

### 5.1.1 Preventive

#### *5.1.1.1 Tumor Surveillance: Genetic Counselling, Whole Body Imaging and Biochemical Testing*

Genetic counselling is the key and the first step taken towards preventative measures amongst suspected familial cases of HNPGLs. The average range of age at diagnosis of hereditary forms of *SDHD* and *SDHB* is less than 40 years (Boedeker et al., 2014; Hensen et al., 2010). Patients with positive family history of known mutation should have that specific mutation tested (Karasek et al., 2013). In asymptomatic cases and incidental findings, commonly seen in HNPGLs, results may vary, as demonstrated in 3 of our patients. It has also become apparent that about 35% of sporadic HNPGLs are due to a germline mutation in these susceptible genes (Guha et al., 2019). Furthermore, germline mutations in *SDHx* also occur in about 30% of HNPGLs that are regarded sporadic due to the absence of a family history, reiterating the theory of "occult familial" cases (Heesterman et al., 2013), as reported in our study. Therefore, patients suspected of heritable HNPGLs (with predictors of risk factors; Figure 3) should undergo genetic analysis first at the *SDHD* and *SDHB* loci, followed by *SDHC*. Additionally, with negative results of above gene mutations and increase in biochemical marker levels, *VHL* and *TMEM127* gene sequencing should be considered (Karasek et al., 2013). If metastatic tumors or multiple abdominal paragangliomas are found without any familial presentation, presence of *SDHB* mutations should be tested first (Guha et al., 2019). Genetic carrier testing should be offered to healthy first-degree relatives including second-degree relatives with *SDHD* and *SDHAF2* which are maternally imprinted. It should be started 5 years before the earliest age of onset in the family (Muth et al., 2018). Since somatic mutations are also detected in 30% of sporadic HNPGLs, mainly involving *VHL, NF1*, *RET* and *HIF2α* genes (Guha et al., 2019), therefore these should be tested. Even though, such genes are rarely associated with multiple HNPGLs.

Whole body imaging is another technique suggested for early detection; it complements genetic testing and plays a vital role in asymptomatic carriers of *SDHx* mutations. Initial suggestions had been made of regular screening for development of tumors as early as 5 to 10 years of age to allow adequate treatment with minimal morbidity (Papaspyrou et al., 2012), however recent recommendations state testing should be started at a later age (Muth et al., 2018). Logically, hereditary carriers of the disease should have a more detailed and frequent imaging work-up in comparison to sporadic cases (Taïeb et al., 2014). Anatomical imaging such as CT or MRI (the preferred technique) always helps with primary localization (Taïeb et al., 2014). It also preferred where there is allergy of CT-contrast media, in pregnant or pediatric patients, and amongst those in whom radiation exposure should be limited (Martucci & Pacak, 2014). Ultrasound has limited utility in non-cervical HNPGLs. CBPGLs and eventually VPGLs can present as incidental findings especially during thyroid gland imaging. Also, it has some role in analyzing vascular tumors like HNPGLs (Chovanec et al., 2021). More importantly, in terms of imaging techniques, the availability of PET/CT has modernized and eased the comfort in which effective management can be based on (Taïeb et al., 2014). 18F-FDOPA PET/CT, which was initially developed to investigate dopaminergic neurotransmission, was found to be a highly sensitive (95% for HNPGLs) and specific (95%–100%) imaging modality for the detection of PGLs, especially HNPGLs (Crona et al., 2017). 18F-FDOPA PET/CT may be performed for confirmation of diagnosis of pheochromocytoma/paraganglioma, staging at initial presentation, restaging and follow-up of patients. It should be mentioned that for SDHx-mutated PPGLs, the practical use of 18F-FDG PET/CT turned out to be a revolution, with 100% and 92% detection rates for primary and metastatic tumor respectively, leading to the 2014 recommendation by the US Endocrine Society Task Force for Pheochromocytoma (Lenders et al., 2014; Santhanam & Taïeb, 2014). Additionally, in SDHx-related syndromes, it is recommended to use 18F-FDG PET/CT in addition to 18F-FDOPA PET/CT (Lenders et al., 2014; Santhanam & Taïeb, 2014). In patients with multiple HNPGLs, we highly recommend annual surveillance with local anatomical imaging (MRI) and functional (PET/CT) radiological investigation every 2-3 years (Papaspyrou et al.,

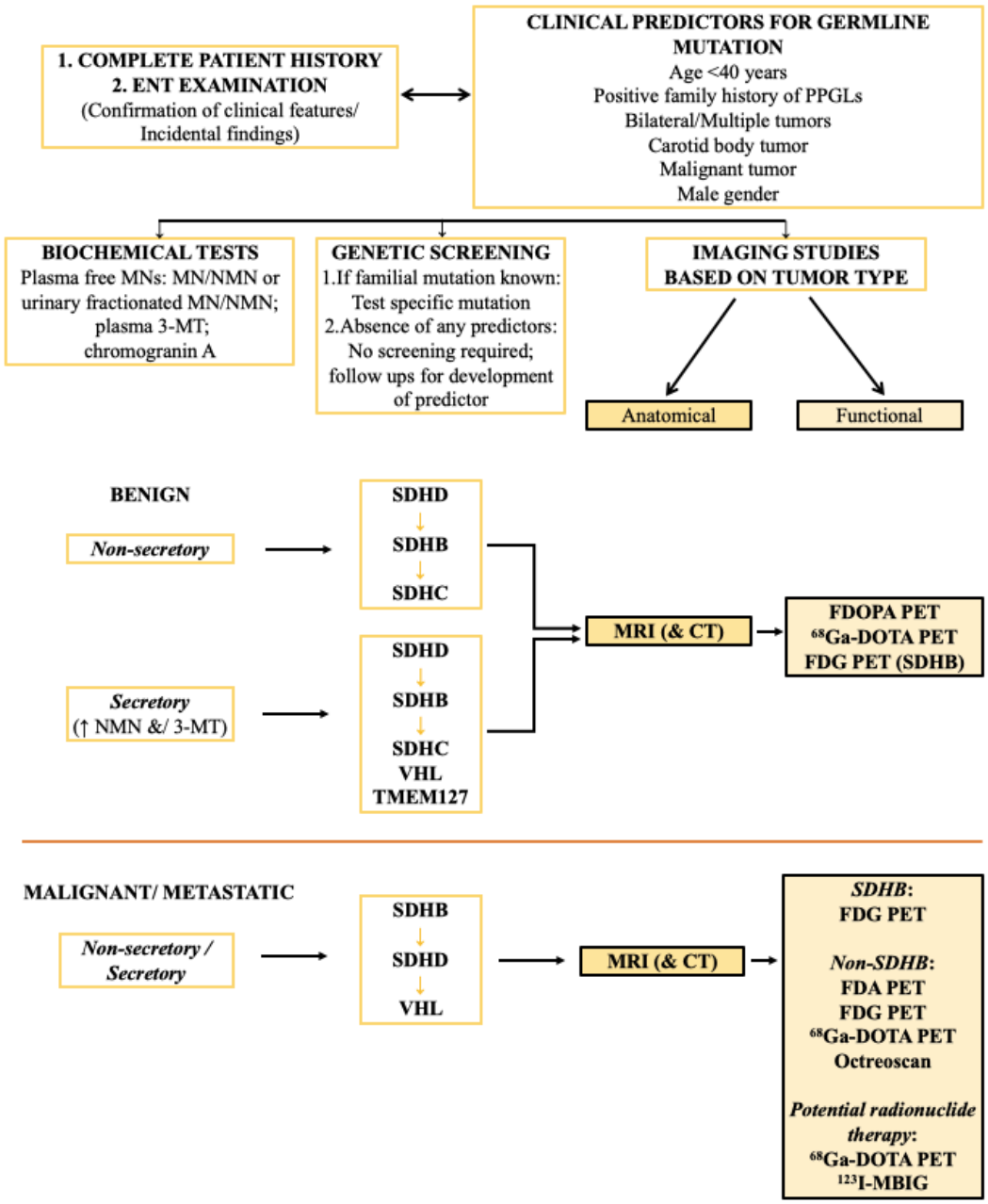
2012). Identification of new tumors in multiple HNPGLs, not only facilitates their detailed evaluation but also helps in changing strategies in management.

Most HNPGLs are biochemically silent, as seen in our patients, only 3-4% are catecholamine-secreting. Plasma metanephrines, normetanephrines and methoxytyramine in particular indicate biochemically active tumors and can be useful for monitoring such patients (van Duinen et al., 2013). In symptomatic or secretory tumors, it can be elevated by 4 times the upper limit (Karasek et al., 2013). Chromogranin A is also released by neuroendocrine tissues along with catecholamines. However, this test is also conducted to evaluate the risk of development of pheochromocytomas in such patients. It is a sensitive and specific diagnostic tool in detecting both familial and sporadic pheochromocytomas. Interestingly, the concentration of plasma chromogranin A also predicts the size of the pheochromocytoma (Hsiao et al., 1990). Even though two of our patients had elevated Chromogranin A levels, all tumors were otherwise biochemically silent and no pheochromocytoma was detected. Therefore, it may show predictive risk value for future tumors.

Lastly, we should reiterate the importance of genetic examination once again, it is not only invaluable in predictability of tumor occurrence in index patients but surveillance in relevant at-risk relatives (Boedeker et al., 2014; Guha et al., 2019, 2021; Neumann et al., 2002; Offergeld et al., 2012; Taïeb et al., 2014; Wasserman & Savargaonkar, 2001). The most valuable application is predicting the risk of tumor development in multiple HNPGLs and/ or familial forms of the disease where family history is negative (Guha et al., 2021). The cost of genetic testing can vary from type of test and insurance coverage amongst different centers worldwide, the main disadvantage being the unavailability of specialized lab facilities in many countries.

We decided to propose an algorithm for screening of index patients and carrier relatives with suspected hereditary forms of HNPGLs (Figure 7). Here, we have decided to include male gender as a risky clinical predictor, since the disease is 3-4 times more common in females. We have also reiterated the importance of the malignant form of the disease.

Figure 7. Proposed algorithm for screening of index patients suspected of hereditary forms of HNPGLs




MET – Metanephrine; NMET – Normetanephrine; 3-MT – 3 -Methoxytyramine

### *5.1.1.2 Malignancy in Multiple HNPGLs*

Diagnosing malignancy in PGLs can be challenging and controversial, since valid histopathologic criteria do not exist (Papaspyrou et al., 2012). We found metastasis in our patient on examination of resected lymph node in a patient with absence of *SDHB* mutation. It has been suggested that immunohistochemistry

staining for SDHB positivity could differentiate SDHx-related PPGLs from other familial syndromes (MEN 2, VHL, NF1) or even sporadic tumors; similar results can be seen for detection of carriers with SDHA germline mutation (Karasek et al., 2013). The pooled incidence for malignant paragangliomas is about 8% in *SDHD* mutation carriers, whilst for *SDHB* can be as high as 41% (Fliedner et al., 2010; van Hulsteijn et al., 2012). Highest risk of malignancy is seen in sinonasal PGLs amongst all head and neck localizations (Rinaldo et al., 2004). Malignancy rates in carotid PGLs rises from 1.51% in unilateral (Guha et al., 2019) to 6-12% in bilateral cases (Robertson et al., 2019). Jugulotympanic and vagal tumors have risks of 5.1% and 6-19% (Rinaldo et al., 2004) respectively.

FDG-PET is recommended in metastatic cases with *SDHB* mutation. Early identification of tumors is of particular importance with *SDHB* due to the risk of metastasis. PGL related metastases occur most frequently in regional lymph nodes (nearly 70%), bones, lungs and liver (Fliedner et al., 2010). Imaging in metastatic tumors is dependent on the type of mutation. 18F-FDG PET/CT should be used for assessment of metastatic PPGLs related to *SDHB*-related tumors (Lenders et al., 2014; Santhanam & Taïeb, 2014; van Berkel et al., 2019), can also be used as an alternative to FDA PET, $^{68}$Ga-DOTA PET or octreoscan for non-SDHB-related PGLs (Martucci & Pacak, 2014). If radionuclide therapy is being considered, then either $^{68}$Ga-DOTA PET or $^{123}$I-MIBG can be considered (Figure 7).

Elevated Norepinephrine can be a marker for metastatic PGLs (Fliedner et al., 2010). Furthermore, In HNPGLs, Chromogranin A correctly predicts the result of paraganglioma surveillance more often in patients with *SDHB* compared with those with *SDHD* (77% vs. 22%, $P = 0.003$) and has less added benefit to standard surveillance (Thompson et al., 2019). Therefore, has a useful negative predictive value when normal in patients with *SDHB* mutation (Thompson et al., 2019). Suggestions such as metastasis to non-neuroendocrine tissues (Lee et al., 2002), *SDHB* mutation carriers and tumors greater than 5 cm in diameter can be significant predicting factors for malignancy (Fliedner et al., 2010; van Hulsteijn et al., 2012). Failure to consent for genetic testing, often seen amongst relatives of index patients, increases the possibilities of underestimating risk of tumor development in asymptomatic carriers. It also does not offer antenatal or invitro mutation analysis

for panel genes of pheochromocytoma/paraganglioma hence creating uncertainty amongst young syndromic patients who decide on parenthood (Guha et al., 2021). This would be beneficial to at least 50% of our patients.

### 5.1.2 Intermediate

#### *5.1.2.1 Wait and Scan Approach*

HNPGLs are very slow growing in nature (1-2mm/year) and predominantly benign in nature (Jansen et al., 2000), thus expected growth would be from 1 to 2cm in a decade. Therefore, the character of these tumors can be followed over time and the 'wait and scan' policy can be used for small tumors or asymptomatic cases with low risk of malignancy. However, this approach should be used with caution in multiple HNPGLs, especially with germline mutation. This can lead to destruction of adjacent structures and irreversible complications; hence these patients should be monitored very closely with regular MRI scans. It has been demonstrated that this approach could not prevent tumor-induced complications in 16% of nongrowing tumors (Jansen et al., 2017). Another disadvantage is that regular follow-up examinations requires patient's compliance.

### 5.1.3 Definitive

#### *5.1.3.1 Surgical Therapy*

The main aim is achieving long-term tumor control with minimal cranial nerve morbidity. The risk of major vascular injury is high for CBPGLs, especially for Shamblin III and even for vagal tumors, it could be as high as 100% (Taïeb et al., 2014). It is recommended that patients should undergo preoperative embolization to reduce perioperative morbidity (Robertson et al., 2019). We believe that preoperative embolization is not strictly indicated in small tumors (e.g. carotid body Shamblin I and tympanic paragangliomas Fisch A), however it depends on clinical judgement. All our patients underwent preoperative embolization before surgery without post-interventional complications. In our cohort of patients, 20 with solitary tumors and 3 with multiple tumors underwent surgery. We operated on 26 tumors (6 CBPGLs, 9 JPGLs, 5 TPGLs, 6 VPGLs), that is 61.9% of totally identified tumors (Guha & Chovanec, 2021).

We used the Infratemporal approach type A (IFTA) in JPGLs, which is well recognized as the best surgical approach for management of JGPGLs as it permits unlimited exposure of the jugular foramen as well as control of the petrous part of internal carotid artery. Although, anterior rerouting of CN VII (a crucial step) improves surgical control of lower cranial nerves and other critical structures, the highest incidence of cranial nerve deficits were seen amongst the patients who underwent surgery for JPGLs (Guha & Chovanec, 2021). A large study reviewed the results of 1084 cases with 1183 preoperative cranial nerve palsies, but surgery actually led to 965 new cranial nerve deficits (Suárez et al., 2013). In advanced cases of tympanojugular tumors, then age and cranial nerve functional status are important. Elderly patients with normal lower cranial nerve status can be allocated to 'wait and scan' or combined subtotal removal of tumor and radiotherapy or radiotherapy only to preserve the function. In younger patients with normal cranial nerve status, Infratemporal approach with preservation of jugular bulb or total removal can be done. These approaches are reiterated by many authors (Fisch, 1978; Green et al., 1994; Shin et al., 2012). Preservation of cranial nerve function would be even more difficult to achieve in multiple HNPGLs; therefore, surgery should never be done in a single stage, thus preventing bilateral cranial nerve deficits and irreversible disabilities (Taïeb et al., 2014). It is recommended to remove the larger tumor first, then the contralateral side can be managed in combination with either surgery or other modalities. We used the same policy in our patients. In patients with jugular tumors, surgical interval of 9 to 12 months should be maintained, since the jugular bulb is usually resected and venous collaterals need to be developed.

One important step in surgery of HNPGLs is performing selective neck dissection (Guha & Chovanec, 2021; Taïeb et al., 2014). This procedure is performed because the tumors are often associated with lymphadenopathy and this leads to proper exposure of tumor as well as improved visualization of critical neurovascular structures; more importantly the presence of lymph node metastases in these tumors cannot be excluded without histopathologic examination. In one of our patients with JGPGL, this step led to the identification of lymph node metastasis that was not suspected both on preoperative clinical examination and imaging

(Guha & Chovanec, 2021). Therefore, this procedure carries a valuable diagnostic purpose.

Cranial nerve morbidity amongst our patients with other HNPGLs was either minimal or well compensated. Long-term results after complete surgical resection of CBPGLs to be excellent, with cure rates reported to be as high as 89% to 100%, well over 90% in VPGLs and 92.5% for tympanic tumors (Kollert et al., 2006; Robertson et al., 2019; Suárez et al., 2013). In jugular tumors, cure rates have been achieved in over 70% (Suárez et al., 2013). Over a period of 1 to 4 years depending on the time of surgery, we achieved local control in 22 out of 23 patients. One patient had planned two-staged surgery for a giant JPGL to prevent postoperative cerebrospinal fluid leakage. However, we performed removal of extradural tumor only. The second stage surgery to remove intradural tumor removal was not realized since the residual tumor regressed on review MRI. Lower cranial nerve as well as facial nerve function were preserved (Guha et al., 2021; Guha & Chovanec, 2021). Deterioration of cranial nerve status was mainly observed in cases of nerve infiltration and encasement by both JPGL and VPGL. Therefore, although tumor type, size and localization should be respected if planning surgery, this should still be considered as frontline in experienced hands.

*5.1.3.2 Radiotherapy*

Radiotherapy is the second most commonly used technique, since paragangliomas are deemed to be radiosensitive. External beam radiation is commonly used for unresectable HNPGLs, with up to 90% 15-year local control rates (Chino et al., 2009; Martucci & Pacak, 2014). Radiosurgery (image-guided radiosurgery or stereotactic surgery) uses a more precise form of therapeutic radiation and almost eliminates side effects seen with conventional radiotherapy. Furthermore, actuarial 10-year progression-free survival can be above 90% (Ibrahim et al., 2017; Krych et al., 2006). This approach could be beneficial with extensive jugular tumors, where some degree of vagus nerve dysfunction exists preoperatively (Netterville & Civantos, 1993) or has a relatively high risk of lower cranial nerve deficit postoperatively (Sen et al., 2001). Alternatively, preoperative radiotherapy maybe used in jugular tumors, where high risk of cranial nerve injuries

is expected, there is possibility of incomplete resection and/or in aggressive behavior of these tumors (Taïeb et al., 2014). Although, comparative analysis showed clinical improvement in patients who underwent Gamma knife radiosurgery versus microsurgical resection in jugular tumors (Gottfried et al., 2004), cranial nerve dysfunction, pre-existing with large tumors (of more than 7cm) or postoperatively, still has a risk of worsening the symptoms (Ibrahim et al., 2017).

Presence of pre-existing nerve palsy should be considered as a negative factor for both radiosurgery or surgical intervention. Similarly, stereotactic radiosurgery also has reported disadvantage of size when treating PGLs. The considered ideal size is less than 3cm. Another major problem is the inability to use the standard stereotactic frame for irradiating CBPGLs. Furthermore, there is no sufficient evidence to justify the use of radiosurgery over surgical resection in cases of secretory HNPGLs (Taïeb et al., 2014). The other consideration is the long-term risk of developing delayed radiation-induced malignancies, which have been reported up to 15 years after radiation when treating benign paragangliomas (Krych et al., 2006). The incidence of radiation-induced fibrosarcoma is approximately 1 in 1000 to 2000 (Lalwani et al., 1993). Other malignancies such as anaplastic astrocytoma (Preissig et al., 1979), malignant peripheral nerve sheath tumor of the vagus nerve (Lekovic et al., 2020) and even brainstem glioblastoma (Na et al., 2015) have also been reported. The main problem is that such malignancies may rise a few decades after treatment, hence it may not be ideal in young patients. Future developments in radiation would be treating multifocal tumors, thus allowing irradiation of 3 or 4 synchronous sites. Another major disadvantage of using radiotherapy is unavailability of direct tumor sampling for genetic evaluation of somatic mutation, which in patients with both positive and negative germline statuses, helps to plan for future treatment in case of recurrence or metastasis. Nonetheless, the higher risk of cranial nerve morbidity that has been reported with open surgery and an operative mortality of 1 in 100, supports the use of this technique even as a frontline modality (Hinerman et al., 2001; Suárez et al., 2013). In multiple tumors, this might be of significant value since it reduces the risk of severe debilitation. However, given the fact that many other factors also play a role in decision making, we recommend to employ this mode of treatment amongst

patients with multiple HNPGLs to treat the contralateral side or as postsurgical salvage therapy with the exception of young patients and secretory tumors. Radiosurgery would be considered in at least 2 of our postoperative patients for the other paragangliomas if tumor growth was noted on (Guha et al., 2021) follow-up scans.

*5.1.3.3 Chemotherapy*

This technique may be used amongst patients with metastatic disease, where it may have palliative effect. For widespread disease with PPGLs, traditionally, chemotherapy with cyclophosphamide, vincristine, and dacarbazine has been used extensively (Adjallé et al., 2009; Martucci & Pacak, 2014), even being observed to be effective in *SDHB* mutations in alleviating symptoms (Martucci & Pacak, 2014), but with no improvement in overall survival (Martucci & Pacak, 2014). Furthermore, many chemotherapeutic agents have been combined and tried (Adjallé et al., 2009), but no definitive conclusions can be made.

5.1.4 Innovative

*5.1.4.1 Targeted Therapy, Radionuclide Therapy and Therapeutic Radiation*

In order to understand the concept of targeted therapy, it is important to understand the mechanism of tumorigenesis in HNPGLs. We discussed the tumorigenic pathways involved in the development of HNPGLs (Table 3). In cluster 1 tumors, glycolysis is activated (Burnichon et al., 2016) and angiogenesis as well as hypoxia are increased (Sobol & Dailey, 1990). Whilst Cluster 2-related tumors include genes that mediate translation initiation, protein synthesis, adrenergic metabolism, neural/neuroendocrine differentiation and abnormal activation of kinase signaling pathways such as RAS/RAF/MAPK and PI3K/AKT/mTOR (Burnichon et al., 2016; Sajid et al., 2007; Vicha et al., 2013). Consequently, pathogenic factors associated with these tumors can be targeted accordingly. Sunitinib, a tyrosine kinase inhibitor, that targets vascular endothelial growth factors which inhibit angiogenesis have shown varying but not very encouraging results even amongst patients with metastasis. Many other possibilities have also been discussed (Martucci & Pacak, 2014).

Radionuclide therapy like somatostatin analogues has been demonstrated in 4 patients with non-metastatic non-resectable progressive PGL1 syndrome where partial response and disease stability was achieved (Zovato et al., 2012). Another option is MIBG treatment, the preferred first treatment for patients with moderately progressing MIBG-avid metastatic PPGLs (Adjallé et al., 2009; Carrasquillo et al., 2012; Martucci & Pacak, 2014). Such treatment is associated with milder side effects and the ease of outpatient treatment as well as patients benefiting from partial responses like reduced symptoms and lowered biochemical levels, the highest recorded rates of complete response is only 15% (Carrasquillo et al., 2012). The use of therapeutic radiation to treat multifocal tumors also brings a promising future, the possibility of treating multiple sites in 1 to 3 outpatient sessions by the use of a combination of antiangiogenics and radiosurgery. This would ideally lead to the disruption of neovasculature and the tumor's supply of oxygen and nutrients. Antiangiogenic therapy has to be administered prior to radiosurgery but the optimal time to initiate this antiangiogenic therapy for radiosensitization in a patient is yet to be determined (Taïeb et al., 2014). However, effectiveness of using such therapeutic options are still very much debatable.

Management of germline mutation differs from somatic mutation in terms of close monitoring of patients in the former cases. Regular annual follow ups with scanning and biochemical tests should be administered for index patients diagnosed with a germline mutation. PET/CT is recommended for 3-5 years follow-up. Predictability of new tumors is not associated with somatic mutation. But, those with sporadic tumors should be re-evaluated for the development of new clinical predictors. If a risk factor evolves, complete genetic evaluation with appropriate whole body imaging is recommended. Both surgery and or radiotherapy can be used with both germline and somatic mutations. Combination of these techniques in patients with multiple tumors and germline mutation have a significant impact on the quality of life. It aids with good tumor control whilst reducing the risk of cranial nerve morbidity. In solitary tumors without clinical predictors of germline mutation, although rare amongst HNPGLs, the minor possibility of malignancy remains.

Furthermore, in patients with *SDHB* mutation, surgery maybe preferred due to the increased risk of malignant tumors and possibility of histopathological examination. Chemotherapeutic agents are used in very rare cases. Targeted therapy would be ideal for cases with germline mutation and multiple tumor localizations in the future.

The normal life expectancy of patients with PPGLs are comparable to the normal population, there is no significant statistical difference (de Flines et al., 2011). This can be attributed to significant improvements in early diagnosis and modernized treatment strategies including improvement of surgical skills and techniques as well as use of lesser invasive techniques. This is in accordance with excellent 10-year survival rates, as already discussed here. However certain factors like metastasis, cranial nerve morbidity due to tumor progression, postsurgical events or *SDHB* mutation should be considered as negative prognostic factors.

# 6. CONCLUSIONS

The modern era in scientific medicine has significantly changed the outlook of management in HNPGLs. Over the decades, new diagnostic, therapeutic and prognostic insights of these tumors have been discovered. Preventative measures represent the gold standard in effectively controlling the disease in index patients and their relatives but requires patient compliance. Invitro and prenatal testing for panel genes of PPGLs may bring new insights to the disease and help reduce the risk of development of the disease, but ethical issues may arise due to incomplete penetrance of the disease. Young age (<40 years), multiple tumors, positive family history, presence of carotid body tumor as well as bilateral presentation should be considered hallmarks of germline mutations in HNPGLs, therefore indicating genetic analysis. Whilst *SDHD* are the most commonly mutated gene in HNPGLs, mutation in *SDHB* carries the highest malignant risk. Our findings from Czech Republic have been significantly different from other European cohorts. We reported higher prevalence of *SDHB* mutation than *SDHD*. We were unable to prove our first hypothesis, since merely 11.5% of examined patients had the *SDHD* mutation. Furthermore, if we studied only Czech patients, then the prevalence further reduces to 8%. Furthermore, we also assumed young age <40 years and/or

the presence of bilateral CBPGLs would have a higher affinity for *SDHD* mutation, which we were unable to prove. We had a total of 5 young patients and 3 (two below the age of 40 years) with bilateral CBPGLs; but only 1 out of 5 young patients was positive for the *SDHD* mutation, and he also had bilateral tumors. As such, germline mutations in *SDHA* and *SDHC* are associated with HNPGLs and sympathetic paragangliomas. *SDHAF2* mutations are associated with HNPGLs. *SDHD* and *SDHB* mutations are associated with HNPGLs, sPGLs and PHEOs, whilst *TMEM127* and *MAX* with PHEOs. Furthermore, germline mutations in *SDHx* also occur in about 30% of HNPGLs that are regarded sporadic due to the absence of a family history, confirming that these HNPGLs may be "occult familial" cases. This formed our last hypothesis for our study. Amongst our patients, we could not determine total occult familial cases; however only 2 patients had *SDHD* germline mutation and 4 had *SDHB* mutation. As discussed previously, somatic mutations in other genes could be detected in another 30% of sporadic HNPGLs, mainly involving *RET*, *VHL, NF1*, *HRAS* and *HIF2α*. In our group of 15 patients that were tested for somatic mutation, only two had positive results. One had *SDHB* mutation; the other had *IDH2* mutation, a rare phenomenon. If PPGLs are found without any syndromic or familial presentation, suspected metastatic tumors should be tested for *SDHB* mutations. In comparison to other countries, our study in the Czech Republic reported a different demographic as well as phenotypic-genotypic pattern.

Although only a fraction of HNPGLs are secretory, it is an important factor with respect to preoperative management of patient and options for definitive treatment. It should be taken into account that elevation of certain biochemical markers can be related to certain mutations. Localization and size of HNPGLs as well as cranial nerve function status play very important roles in decision for treatment. The 'wait and scan' approach, although been popularized due to the slow growing nature of these tumors, carries a risk of irreversible damage from tumor infiltration. Preoperative embolization is strongly recommended for those undergoing surgery. Multiple bilateral HNPGLs should never be operated in a single stage as consequences of multiple or bilateral cranial nerve morbidity can be catastrophic. Diagnosing malignancy in HNPGLs can be challenging in the absence

of defined histopathological criteria, therefore lymph node dissection should be considered even in the absence of clinical and radiological signs of metastasis. Immunohistochemistry staining can be used as an adjunctive examination. Combination therapy should be used in multiple HNPGLs where indicated, to reduce the risk of morbidity. If radiotherapy is being considered as frontline therapy, it must be used with caution in young patients or with hereditary tumors. In secretory tumors, this technique is inadvisable. Cranial nerve dysfunction in association with tumor encasement is a negative prognostic factor for both surgery and radiotherapy. Whilst molecular examination predicts phenotype, inheritance and/or risk of development of tumor, it cannot be used to delineate tailored therapy according to mutation type. Therefore, modalities such as gene targeted therapy although shows a massive potential due to the versatile tumorigenic pathways of the disease, is of little use in current clinical practice.

Longstanding follow up should be done in all cases to facilitate the early detection of recurrent or new tumors and status of cranial nerve function. Long-term survival rates of patients with HNPGLs are comparable to general population, with the exception of few cases with poor prognostic factors. We were able to present a significant number of HNPGLs cases within a restricted timeline, but pitfalls of the study include unavailability of data of relatives of index patients and the complex process of availing genetic counselling and sequencing for the patients within defined time limits. We recommend to continue the study to achieve results of a 10-year long-term follow up and reassess the frequency of mutation amongst patients with HNPGLs in Czech Republic with the advent of new patients and identification of carrier status amongst family members.

# 7. SUMMARY

- There is a deficit in cohort studies reporting phenotypic-genotypic characteristics of head and neck paragangliomas (HNPGLs) in Czech Republic, leading to a discrepancy in European statistics. Furthermore, genetics of these tumors plays a vital role in early management of the index patients and their relatives. Therefore, we based our study on these theories.
- Our systematic review showed that 10 genes when mutated can lead to hereditary forms of the disease. *SDHD* was the most commonly mutated gene followed by *SDHB* and *SDHC*.
- We assessed 30 patients (11 males; 19 females) of 34-80 years of age at our clinic between 2016 and 2021. Twenty-eight patients were of Czech origin, one patient was Hungarian and the other was of Polish origin. A total of 42 HNPGLs were found amongst 24 patients with solitary tumors and 6 patients with multiple tumors. Five tumors were incidental findings. According to localization of tumors, we found 31% JPGLs, 26% CBPGLs, 24% VPGLs and 19% TPGLs. Three other PGLs were also confirmed on PET/CT imaging.
- In terms of clinical predictors for hereditary form of the disease, only 2 female patients had positive family history, one was of Czech origin with solitary tumor and the other was a Polish patient with multiple tumors. Five patients presented below the age of 40 years old. Three patients had bilateral carotid body tumors, two of whom were below 40 years of age.
- Genetic examination was performed on biological samples collected from patients, who consented. Germline mutation analysis was done on 26 (*SDHD* in 26; other *SDHx* and non-*SDHx* in 19) patients. Somatic mutation was excluded in 15 patients. Our findings of genetic sequencing show that in comparison to other European countries, Czech Republic has a lower frequency of *SDHD* mutation. Only 11.5% of all our examined patients were positive for this mutation, with a further reduction to 8% amongst Czech patients only. Out of 3 patients with bilateral carotid body tumors, only 1 had *SDHD* mutation. Only 50% of our patients under 40 years of age with bilateral carotid body tumors had *SDHD* germline mutation, and none

of the other 3 young patients had the mutation. *SDHD*-related occult familial cases were seen in only 8.3% of the patients. Four (3 females with solitary tumors; 1 male with multiple tumors) patients with negative family history were diagnosed with *SDHB* mutation and benign tumors. In comparison to PHEOs and HNPGLs, extra-adrenal sPGLs have long been known to have a greater predisposition to malignancy with *SDHB* mutation. This corresponds to our findings. Two pitfalls of the study were the incapability to determine the presence of non-*SDHD* mutation in all 26 patients and the inability to test relatives of index patients. However, we have proposed an algorithm for screening of index cases suspected of hereditary form of the disease.

- Results from somatic mutation analysis revealed a rare mutation of *IDH2* in a male patient with solitary CBPGL as well as *SDHB* mutation in another male patient with JPGL. None of these tumors were malignant.
- A total of 23 (20 with solitary tumors; 3 with multiple tumors) patients underwent surgery following preoperative embolization. Multistep procedure was performed for patients with multiple tumors. Amongst 26 operated tumors, only one had malignant variant with lymph node metastasis. Cranial nerve morbidity amongst our patients with other HNPGLs was either minimal or well compensated. Deterioration of cranial nerve status was mainly observed in cases of nerve infiltration and encasement by the tumor. Over a period of 1 to 4 years depending on the time of surgery, we achieved local control in 22 out of 23 patients. One patient had residual tumor but with signs of shrinkage. The remaining patients were allocated to 'wait and scan' approach either due to age, medical comorbidity or patient's choice. Only 1 patient died due to very advanced disease, declination of treatment and multiple cranial nerve palsies. Radiotherapy would be considered in combination with surgery for our other patients with multiple HNPGLs.
- In conclusion, our study shows that the demographic and phenotypic-genotypic patterns of HNPGLs in Czech Republic are different from other countries. We recommend the use of a more comprehensive

multidisciplinary management protocol in all centers that treat patients with HNPGLs. Testing new index patients, their relatives and long-term follow-up should be carried out with the aims to re-assess the pattern, compare investigative and treatment protocols as well as determine prognostic outcomes in such patients.

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## 9. AUTHOR'S RESEARCH FINDINGS THAT FORMED THE BASIS OF THE DISSERTATION WORK

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SUPPLEMENTARY TABLE 1. NGS PANEL GENES FOR PPGLS

| Gene | Name | ID |
|---|---|---|
| ABAT | 4-Aminobutyrate Aminotransferase | NM_020686.6 |
| ACLY | ATP citrate lyase | NM_001096.2 |
| ACO1 | Aconitase 1, Soluble | NM_001362840.1 |
| ACO2 | Aconitase 2, Mitochondrial | NM_001098.2 |
| AKT1 | V-Akt Murin Thymoma Viral Oncogene Homolog 1 | NM_005163.2 |
| ALDH5A1 | Aldehyde Dehydrogenase 5 Family, Member A1 | NM_170740.1 |
| ALK | Anaplastic Lymphome Receptor Tyrosine Kinase | NM_004304.5 |
| ARID2 | At Rich Interactive Domain 2 (Arid, Rfx-Like) | NM_152641.3 |
| ARNT | Aryl Hydrocarbon Receptor Nuclear Translocator | NM_001197325 |
| ATM | Ataxia Teleangiectasia Mutated | NM_000051.3 |
| ATRX | Alpha Thalassemia/Mental Retardation Syndrome X-Linked | NM_000489.5 |
| BAP1 | BRCA1-Associated Protein 1 (Ubiquitin Carboxy-Terminal Hydrolase) | NM_004656.3 |
| BRAF | B-Raf Proto-Oncogene, Serine/Threonine Kinase | NM_004333.5 |
| CDH1 | Cadherin 1 | NM_004360.5 |
| CDKN2A | Cyclin Dependent Kinase Inhibitor 2A | NM_000077.4 |
| CDKN2B | Cyclin Dependent Kinase Inhibitor 2B | NM_004936.4 |
| CREB1 | Camp Responsive Element Binding Protein 1 | NM_134442.4 |
| CS | Citrate Synthase | NM_004077.2 |
| CSDE1 | Cold Shock Domain Containing E1 | NM_001242891.1 |
| D2HGDH | D-2-hydroxyglutarate dehydrogenase | NM_152783.5 |
| DLAT | Dihydrolipoamide S-Acetyltransferase | NM_001931.4 |
| DLST | Dihydrolipoamide S-Succinyltransferase (E2 component of 2-oxo-glutarate complex) | NM_001933.5 |
| DNMT3A | DNA methyltransferase 3 alpha (DNMT3A) | NM_175629 |
| EGLN1 | Egl-9 Family Hypoxia-Inducible Factor 1 | NM_022051.2 |
| EGLN3 | Egl-9 Family Hypoxia-Inducible Factor 3 | NM_022073.3 |
| EPO-R | Erythropoietin Receptor | NM_000121.3 |
| EZH2 | Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit | NM_004456.4 |
| FGFR1 | Fibroblast Growth Factor Receptor 1 | NM_001174067.1 |
| FGFR2 | Fibroblast Growth Factor Receptor 2 | NM_001320658.1 |

| | | |
|---|---|---|
| FGFR3 | Fibroblast Growth Factor Receptor 3 | NM_001163213.1 |
| FH | Fumarate Hydratase | NM_000143.3 |
| FHIT | Fragile Histidine Triad Gene | NM_001166243.2 |
| GAD1 | Glutamate Decarboxylase 1 (Brain, 67kda) | NM_000817.3 |
| GAD2 | Glutamate Decarboxylase 2 (Pancreatic Islets And Brain, 65kda) | NM_001134366.2 |
| GLS2 | Glutaminase 2 (Liver, Mitochondria) | NM_001280796.1 |
| GLUD1 | Glutamate Dehydrogenase 1 | NM_005271.5 |
| GLUD2 | Glutamate Dehydrogenase 2 | NM_012084.3 |
| GNAS | GNAS Complex Locus | NM_000516 |
| GOT1 | Glutamic-Oxaloacetic Transaminase 1, Soluble | NM_002079.3 |
| GOT2 | Glutamic-Oxaloacetic Transaminase 2, Mitochondrial | NM_002080.4 |
| GPT1 | Glutamic-Pyruvate Transaminase (Alanine Aminotransferase) | NM_005309.3 |
| GPT2 | Glutamic Pyruvate Transaminase (Alanine Aminotransferase) 2 | NM_133443.4 |
| H3F3A | H3 histone, family 3A | NM_002107.5+5UTR |
| HIF1A | Hypoxia Inducible Factor 1, alpha subunit (basic helix-loop-helix transcription factor) | NM_001530.4 |
| HIF1AN | Hypoxia-inducible factor 1, alpha subunit inhibitor | NM_017902.2 |
| HIF2A (EPAS1) | Hypoxia Inducible Factor 2, Alpha Subunit | NM_001430.5 |
| HMOX1 | Hemeoxygenase type (Decycling) 1 | NM_002133.2 |
| HMOX2 | Hemeoxygenase type (Decycling) 2 | NM_001286270.1 |
| H-RAS | Harvey Rat Sarcoma Viral Oncogene Homolog | NM_001130442.2 |
| IDH1 | Isocitrate Dehydrogenase 1 | NM_005896 |
| IDH2 | Isocitrate Dehydrogenase 2 | NM_002168 |
| IDH3A | Isocitrate Dehydrogenase 3 (NAD+) Alpha | NM_005530.3 |
| IDH3B | Isocitrate Dehydrogenase 3 (NAD+) Beta | NM_006899.5 |
| IDH3G | Isocitrate Dehydrogenase 3 (NAD+) Gamma | NM_004135.4 |
| IREB2 | Iron-Responsive Element Binding Protein 2 | NM_001320942.1 |
| JAG1 | Jagged 1 | NM_000214.3 |
| JAK2 | Janus Kinase 2 | NM_001322194.1 |
| JMJDIC | Jumonji Domain Containing 1C | NM_032776.3 |
| JUN | Jun Proto-Oncogene (v-jun Avian Sarcoma Virus 17 Oncogene Homolog) | NM_002228.3 |
| KDM2B | Lysine (K)-Specific Demethylase 2B | NM_032590.5 |
| KIF1Bß | Kinesin Family Member 1b | NM_015074.3 |

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|---|---|---|
| KIT | V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog | NM_000222.2 |
| KMT2D | Lysine Methyltransferase 2D | NM_003482.3 |
| K-RAS | Kirsten Rat Sarcoma Viral Oncogene Homolog | NM_033360.3 |
| L2HGDH | L-2-Hydroxyglutarate Dehydrogenase | NM_024884.3 |
| LDHA | Lactate Dehydrogenase A | NM_001165414.1 |
| LDHAL6A | Lactate Dehydrogenase A-Like 6A | NM_001144071.1 |
| LDHAL6B | Lactate Dehydrogenase A-Like 6B | NM_033195.2 |
| LDHB | Lactate Dehydrogenase B | NM_002300.7 |
| LDHC | Lactate Dehydrogenase C | NM_002301.4 |
| LDHD | Lactate Dehydrogenase D | NM_153486.3 |
| MAML3 | Mastermind-Like 3 | NM_018717.5 |
| MAX | Myc Associated Factor X | NM_002382.4 |
| MDH2 | Malate Dehydrogenase 2 | NM_005918.4 |
| ME1 | Malic Enzyme 1, NADP(+)-Dependent, Cytosolic | NM_002395.6 |
| ME2 | Malic Enzyme 2, NAD(+)-Dependent, Mitochondrial | NM_002396.5 |
| ME3 | Malic Enzyme 3, NADP(+)-Dependent, Mitochondrial | NM_001014811.1 |
| MERTK | MER Proto-Oncogene, Tyrosine Kinase | NM_006343.2 |
| MET | MET Proto-Oncogene, Receptor Tyrosine Kinase | NM_001127500.3 |
| MHD1 | Malate Dehydrogenase 1, NAD (Soluble) | NM_001199111.1 |
| mTOR | Mechanistic Target Of Rapamycin (Serine/Threonine Kinase) | NM_004958.3 |
| MYCN | v-MYC avian myelocytomatosis viral oncogene neuroblastoma derived homolog | NM_005378.6 |
| NF1 | Neurofibromin 1 | NM_001042492.2 |
| NGFR | Nerve Growth Factor Receptor | NM_002507.4 |
| NOTCH1 | Notch (Drosophila) homolog 1 (translocation-associated) | NM_017617.4 |
| N-RAS | Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog | NM_002524.5 |
| OGDH | Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) | NM_002541.3 |
| OGDH | Oxoglutarate (Alpha-Ketoglutarate) Dehydrogenase (Lipoamide) | NM_002541.4 |
| OGDL | Oxoglutarate (Alpha-Ketoglutarate) Dehydrogenase (Lipoamide) | NM_002541.4 |
| PC | Pyruvate Carboxylase | NM_022172.2 |
| PCK1 | Phosphoenolpyruvate Carboxykinase 1 (Soluble) | NM_002591.4 |
| PCK2 | Phosphoenolpyruvate Carboxykinase 2 (Mitochondrial) | NM_004563.3 |
| PDGFRA | Platelet-Derived Growth Factor Receptor, Alpha Polypeptide | NM_006206.6 |

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|---|---|---|
| PDHA1 | Pyruvate Dehydrogenase (Lipoamide) Alpha 1 | NM_001173454.1 |
| PDHA2 | Pyruvate Dehydrogenase (Lipoamide) Alpha 2 | NM_005390.4 |
| PDK1 | Pyruvate Dehydrogenase Kinase, Isozyme 1 | NM_002610.4 |
| PHD2/EGLN1 | Egl-9 Family Hypoxia-Inducible Factor 1 | NM_022051.2 |
| PIK3CA | Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha | NM_006218.3 |
| PRKAR1A | Protein kinase cAMP-dependent type I regulatory subunit alpha | NM_212471.2 |
| PTEN | Phosphatase And Tensin Homolog | NM_000314.7 |
| PTPN5 | Protein Tyrosine Phosphatase, Non-Receptor Type 5 (Striatum-Enriched) | NM_001278236.1 |
| RET | Ret Proto-Oncogene | NM_020975.6 |
| SART1 | Squamous Cell Carcinoma Antigen Recognized By T cells | NM_005146.4 |
| SDHA | Succinate Dehydrogenase Complex, Subunit A | NM_004168.4 |
| SDHAF2 | Succinate Dehydrogenase Complex Assembly Factor 2 | NM_017841.2 |
| SDHAF3 | succinate dehydrogenase complex assembly factor 3 | NM_020186.2 |
| SDHB | Succinate Dehydrogenase Complex, Subunit B | NM_003000.2 |
| SDHC | Succinate Dehydrogenase Complex, Subunit C | NM_003001.3 |
| SDHD | Succinate Dehydrogenase Complex, Subunit D | NM_003002.4 |
| SETD2 | SET domain containing 2 | NM_014159.6 |
| SIRT1 | Sirtuin 1 | NM_012238.4 |
| SLC25A11 | Solute Carrier Family 25 Member 11 (SLC25A11) | NM_003562.5 |
| SUCLG1 | Succinate-Coa Ligase, Alpha Subunit | NM_003849.4 |
| SUCLG2 | Succinate-Coa Ligase, GDP-Forming, Beta Subunit | NM_003848.3 |
| SUCLG2 | Succinate-Coa Ligase, GDP-Forming, Beta Subunit | NM_003848.3 |
| TCF4 | transcription factor 4 (TCF4) | NM_001083962.1 |
| TERT | telomerase reverse transcriptase | NM_198253.2 |
| TET1 | tet methylcytosine dioxygenase 1 | NM_030625.3 |
| TET2 | tet methylcytosine dioxygenase 2 | NM_001127208.2 |
| TMEM 127 | Transmembrane Protein 127 | NM_001193304.2 |
| TP53 | Tumor protein p53 | NM_001276760.1 |
| UBTF | upstream binding transcription factor (UBTF) | NM_014233.3 |
| VHL | Von Hippel-Lindau Tumor Suppressor, E3 Ubiquitin Protein Ligase | NM_000551.3 |